DETERMINATION OF PHYLLOQUINONE AND MENAQUINONES IN FOODS BY HPLC

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ACADEMIC DISSERTATION

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ABSTRACT

High-performance liquid chromatographic (HPLC) methods for determining vitamin K contents in oils and margarines, vegetables, fruits and berries, cereals and animal products were developed. These methods were applied to analysing phylloquinone and menaquinone in a total of 87 food items. In addition, the 2´,3´-dihydrovitamin K_1 contents of a few margarines and hydrogenated oils were determined, and variation in the phylloquinone contents of plant products were investigated.

The quantification of vitamin K compounds was made after solvent extraction, which was optimised separately for each food group, and chromatographic purification. In the case of animal products with high fat contents, lipase treatment was used as an extra purification step. The phylloquinone contents of plant products were analysed by HPLC with electrochemical detection, whereas fluorescence detection after reduction with metallic zinc was applied for determination of animal products. The identification of menaquinones was confirmed with LC-MS.

Phylloquinone was the dominant vitamin K form in foods analysed here; dihydrovitamin K_1 and menaquinones probably play no significant role in vitamin K nutrition in Finland. The highest phylloquinone contents were found in green vegetables, oils and margarines; a remarkable variation was observed in these items. There are several possible reasons, such as genetic factors and processing conditions, for this. In the case of animal products this study concentrated mainly on the developing a validated and documented analytical method for determining their phylloquinone and menaquinone contents. Thus, only a few items were analysed. The vitamin K contents of meat, fish and dairy products were generally low and long-chain menaquinones were found only in livers, cheese and soured whole milk.

The estimated average daily intake of vitamin K was 120 μg/day, of which 30% is derived from oils and margarines, 35% from various vegetables and the rest from cereals, fruits, berries and animal products. Because a fairly high variation especially in the consumption of vegetables among individuals is to be expected, the daily dietary intake of vitamin K may vary considerable.

PREFACE

This study on vitamin K was conducted at the Department of Applied Chemistry and Microbiology, University of Helsinki during the years 1996-2000. This academic dissertation is done under the 'Applied Bioscience – Bioengineering, Food & Nutrition, Environment' (ABS) program of the Finnish Graduate School.

I wish to express my sincere gratitude to my supervisor, Professor Vieno Piironen for introducing me to food composition studies and for suggesting the subject of this work. Her advice and support has been vital during these years. I would also like to thank her for reading my manuscript and her constructive criticism. I thank Dr. Pirjo Mattila and Dr. Liisa Vahteristo for reading the manuscript with thought, and for giving useful comments and suggestions. They were both excellent examples to follow in my pursuit of a doctorate and sources of know-how during my academic career.

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Finally, my dearest thanks go to my family, my husband Tommi and our cheerful daughter Venla. You, who are the most important ones in my life, gave me force to write this thesis.

Vantaa, December 2000

Terhi Koivu-Tikkanen

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by their Roman numerals I-V.

I Piironen, V., Koivu, T., Tammisalo, O. & Mattila, P. 1997. Determination of phylloquinone in oils, margarines and butter by high-performance liquid chromatography with electrochemical detection. Food Chem. 59:473-480.

II Koivu, T., Piironen, V., Henttonen, S. & Mattila, P. 1997. Determination ofphylloquinone in vegetables, fruits, and berries by high-performance liquid chromatography with electrochemical detection. J.Agric. Food Chem. 45:4644-4649.

III Koivu, T., Piironen, V. & Mattila, P. 1998. Phylloquinone (vitamin K_1) in cereal products. Cereal Chem.75:113-116.

IV Koivu, T., Piironen, V., Lampi, A.-M. & Mattila, P. 1999. Dihydrovitamin K_1 in oils and margarines available in Finland. Food Chem. 64:411-414.

V Koivu, T., Ollilainen, V. & Piironen, V. 2000. Determination of phylloquinone and menaquinones in animal products with fluorescence detection after post-column reduction with metallic zinc. J. Agric. Food Chem. (in press).

Contribution of the author to papers I to V:

I. The author performed most part of the vitamin K determinations and was responsible for analytical data. She took part to the finishing of the manuscript.

II-V. The author participated in planning the study, performed most part of the vitamin K determinations and was responsible for analytical data. She was the main author of the papers II-V.

CONTENTS

LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
APCI	atmospheric pressure chemical ionization
ASE	accelerated solvent extraction
CEN	European Committee for Standardization
CV	coeffiicient of variation
$E^{1\%}_{1cm}$	the absorbance of a solution containing one gram per 100 mL contained in a cell having an absorption path of one cm
EC	electrochemical
em	emission
ex	excitation
FL	fluorescence
Gla	γ-carboxyglutamic acid
Glu	glutamic acid
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
HPLC	high-performance liquid chromatography
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC-IUB	International Union of Pure and Applied Chemistry and International Union of Biochemistry
LC-MS	liquid chromatography – mass spectrometry
MK-n	menaquinone (n = number of prenyl groups in side chain)
MS	mass spectrometry
MSPD	matrix solid-phase dispersion
m/z	mass – charge – ratio
NaAc	sodium acetate
PIVKA	protein induced by vitamin K absence
RDA	recommended dietary allowance
RNI	reference nutrient intake
SPE	solid-phase extraction
TBAP	tetrabutylammoniumphosphate
ucOC	undercarboxylated osteocalcin
UV	ultraviolet

1 INTRODUCTION

Vitamin K was discovered by Henrik Dam in 1929 as an antihemorrhagic factor in chicks. Few years later it was shown that the haemorrhagic disease was due to the absence of prothrombin activity in the plasma. Alfalfa and putrefied fish meal were observed to be good sources of this new vitamin. Vitamin K was isolated simultaneously from both sources as vitamin K_1 and vitamin K_2, respectively. After the identification of its structures for several decades vitamin K received less attention than other fat-soluble vitamins. The main reasons for this were its wide distribution in foods and the small requirement for this vitamin in humans. Since discovering a new amino acid, γ-carboxyglutamic acid (Gla) and the role of vitamin K as a cofactor in its posttranslational synthesis during the 1970s vitamin K has been a subject of active research (Parrish, 1980; Olson, 1994).

These findings led to the isolation of new vitamin K-dependent proteins from various tissues. Thus the functions of vitamin K was expanded from its historical role in blood coagulation at least to bone metabolism (Shearer, 1995). The exact functions of newly discovered vitamin K proteins are not known except for the function of osteocalcin in the regulation of bone growth, it is also unclear what are the best markers for the evaluation of vitamin K status. Although it is generally assumed that the deficiency of vitamin K is probably more common than previously believed, more research is still needed before the present dietary recommendations can be re-evaluated (Vermeer et al., 1998).

On the other hand, vitamin K has an effect on the efficiency of anticoagulant drugs, such as warfarin. Their function is based on the antagonizing the metabolism of vitamin K, which results in a failure to synthesize Gla. It is widely assumed that a dietary vitamin K – warfarin interaction exists although a dose-response of vitamin K on the effect of warfarin anticoagulation has not yet been established. However, for patients on warfarin it is important to a have constant dietary vitamin K intake (Booth and Centurelli, 1999).

The natural forms of vitamin K are phylloquinone (vitamin K_1) and menaquinones (vitamin K_2). The best sources of phylloquinone, which is synthesised by plants, are dark-green vegetables and vegetable oils (Booth et al., 1996a; Booth and Suttie, 1998). Menaquinones are of microbial origin and can be find both in animal products and in the intestine. The biovailability of intestinal menaquinones in vitamin K nutrition is, however, unclear (Vermeer et al., 1995). Whereas the quality of data on phylloquinone contents in plant foods has

increased in the 1990s, information about menaquinones is still very limited. Most food composition tables do not include values for vitamin K, thus in order to further understand the nutritional role of vitamin K, more information of vitamin K-active compounds, including phylloquinone and menaquinones, in foods is needed.

Although the introduction of high-performance liquid chromatography (HPLC) has facilitated vitamin K analysis, the determination of various K vitamers is still a great challenge. Electrochemical and fluorescence detectors after post-column reduction provide enough sensitivity and selectivity for analysing phylloquinone in plant products (Fauler et al., 2000). Due to low vitamin K concentrations in animal products, further improvements in detection systems are needed. In addition, extraction and purification methods have to be developed so that they are more useful before reliable vitamin K data can be produced. Thus, before this study began there was a clear need for validated and carefully documented methods for analysing phylloquinone and menaquinones as well as for new data on their occurrence in foods.

This thesis consists of three parts: The first part of the thesis reviews the vitamin K literature mainly concentrating on the methodology of vitamin K analysis in foods. The second part is a review of the experimental part, in which the HPLC methods for analysing phylloquinone and menaquinones in various food items were developed. In addition, vitamin K contents for several food items analysed in this study are summarised. The last section consists of the five original papers that form the basis of this study.

2 LITERATURE REVIEW

2.1 Nomenclature and chemistry of vitamin K

Vitamin K is the generic term for a family of compounds that act as a cofactor in the post-translational synthesis of γ-carboxyglutamic acid (Gla). All K vitamers are derivatives of the same 2-methyl-1,4-naphthoquinone structure; the molecules are characterised by the number of isoprene units in the lipophilic side chain (Parrish, 1980; Lambert and de Leenher, 1992). The nomenclature of compounds possessing vitamin K activity has been modified a number of times since the discovery of vitamin K. The nomenclature used in the present thesis is based on the recommendations of The International Union of Pure and Applied Chemistry and International Union of Biochemistry (IUPAC-IUB, 1992).

Vitamin K exists naturally in two forms; as phylloquinone (vitamin K_1) and menaquinones (vitamin K_2). The chemical structure of phylloquinone (Figure 1A) is 2-methyl-3-phytyl-1,4-naphthoquinone having a phytyl group with one double bond as a side chain. It is synthesised by plants whereas menaquinones, 2-methyl-3-$(prenyl)_n$ -1,4-naphthoquinone (MK-n, Figure 1B), are of microbial origin. They are named according to the number of prenyl groups (up to 13) in the unsaturated side-chain (Lambert and de Leenher, 1992). Phylloquinone exists naturally only in the *trans* form, and the *all-trans* configuration is also the most common one for menaquinones. *Cis-trans* isomers, which are formed during UV light exposure or synthetic production of vitamin K, are considered to have low bioactivity (Parrish, 1980; Indyk, 1988a).

Menadione, formerly known as vitamin K_3, is a synthetic form; its structure is 2-methyl-1, 4-naphthoquinone (Figure 1C). It has no vitamin K activity but it can be alkylated enzymatically to MK-4 in animal tissues (Dialameh et al., 1971). A number of other related compounds with varying activities have been synthesised, for example 2´,3´-dihydrovitamin K_1 (Figure 1D) and $K_{1(25)}$ (Figure 1E). The former is formed from phylloquinone during the hydrogenation of oils (Davidson et al., 1996) whereas $K_{1(25)}$ is produced by the substitution of a 25-carbon side chain to menadione. Both these forms are commonly used as internal standards in vitamin K analysis (Booth and Sadowski, 1997).

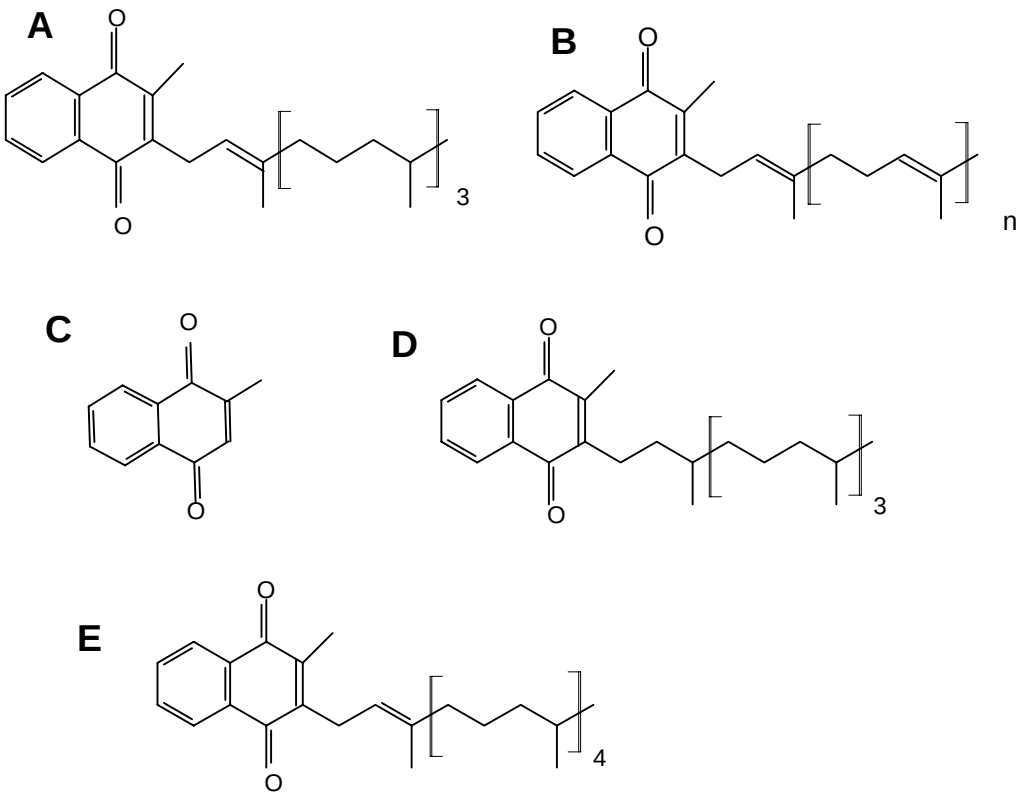

Figure 1. Chemical structures of phylloquinone (A), menaquinone (B), menadione (C), dihydrovitamin K_1 (D) and $K_{1(25)}$ (E).

Due to the close structural relationship of different vitamin K forms, most of their chemical and physical properties are similar. Phylloquinone is liquid at room temperature whereas menadione and menaquinones are solids (Parrish, 1980). K vitamers are insoluble in water, slightly soluble in alcohol and readily soluble in non-polar organic solvents, for example in *n*-hexane, ether, and chloroform. They are sensitive to light and alkaline conditions, but stable in slightly acidic media and under oxidising conditions. They also have relatively high thermostability (Lambert and de Leenher, 1992). All K vitamers have an ultraviolet spectrum characteristic of the naphthoquinone nucleus with four distinct peaks between 240 nm and 280 nm. The extinction coefficient decreases as the length of side-chain increases (Parrish, 1980).

The main metabolic reaction of vitamin K is epoxidation of the double-bond between carbons 2 and 3 of the naphthoquinone ring resulting in vitamin K 2,3-epoxide. This form is

reduced in vitamin K cycle (Figure 2) to quinone form and further to hydroquinone form; the detection of vitamin K with fluorescence is based on the latter reaction. These reactions are reversible and hydroquinone can be reoxidised back to quinone form. Other vitamin K reactions include saturation of the double-bonds, phosphorylation and alkylation (Mayer and Isler, 1971; Parrish, 1980).

2.2 Vitamin K nutrition

2.2.1 Absorption and bioavailability

Vitamin K is absorbed mainly from the small intestine into the lymphatic system; optimal absorption needs the presence of both bile acids and pancreatic juice as reviewed by Olson (1984). It has been shown that under normal conditions vitamin K is moderately well (40 to 70%) absorbed from the small intestine (jejunum and ileum), but very poorly from the colon (Olson, 1984). The source of vitamin K seems to influence the efficiency of absorption; both Gijsbers et al. (1996) and Garber et al. (1999) have observed that circulating levels of phylloquinone were significantly higher after ingestion of the pharmaceutical concentrate than after spinach. Garber et al. (1999) did not find any differences between different vegetables (spinach, broccoli and Romain lettuce) or between raw and cooked broccoli. The fat content of a meal has been shown to influence absorption of both phylloquinone from vegetable and MK-4 from pharmaceutical concentrate (Gijsbers et al., 1996; Uematsu et al., 1996; Garber et al., 1999). On the other hand, Booth et al. (1999a) did not find any differences in the bioavailability of phylloquinone from oil or broccoli when consumed together with mixed diet. Very little is known about the absorption of dietary menaquinones although an effect of orally ingested menaquinones on prothrombin time has been demonstrated (Conly and Stein, 1993).

The role of menaquinones produced by the intestinal microflora on maintaining vitamin K status is unknown; there are arguments both for and against their absorption (Conly and Stein, 1992; Shearer, 1992; Lipsky, 1994; Suttie, 1995; Vermeer et al., 1995). Suttie (1995) and Vermeer et al. (1995) suggested that the gut menaquinones might have little significance because most of them are located in bacterial membranes and thus are probably not available for absorption. In addition, studies of Ichihashi et al. (1992) and Groenen-van Dooren et al. (1995) have shown that absorption of menaquinones from the colon in the ab-

sence of bile acids is extremely poor in rats. Furthermore, Lipsky suggested in his review (1994) that bacterial production of menaquinones is unimportant because both humans and rats can have nutritional deficiencies of vitamin K. On the other hand, high menaquinone concentrations found in liver indicate that some absorption of menaquinones exists (Shearer, 1992). It has been assumed that the absorption of bacterial menaquinones is possible via backwash past the ileocecal valve into the ileum where bile acids are present (Conly and Stein, 1992) or by a passive diffusion process from the colon (Shearer, 1992). Although Conly et al. (1994) have proved the positive effect of colorectally ingested menaquinones on prothrombin time, very little direct evidence for the absorption of intestinal menaquinones in humans exists.

Absorbed vitamin K is transported primarily via the lymph in chylomicrons to the liver, which is often thought to be the largest storage organ for vitamin K (Shearer, 1995). Phylloquinone and MK-4 are recovered in most of human tissues although the long-chain menaquinones probably comprise most of the liver's store (Usui et al., 1990; Shearer, 1995; Thijssen and Drittij-Reijnders, 1996). In rats high MK-4 concentrations are found from nonhepatic organs after high phylloquinone intakes. This is probably due to conversion of dietary phylloquinone to MK-4; it is known that this conversion is not dependent on gut bacteria although its exact route is still unclear (Thijssen and Drittij-Reijnders, 1994; Thijssen et al., 1996; Davidson et al., 1998; Ronden et al., 1998). On the other hand, MK-4 may be converted to MK-8 via an unknown metabolic pathway (Koivu-Tikkanen et al., 2000). Studies cited above as well as findings of Huber et al. (1999) suggest spesific metabolic roles both to MK-4 and MK-6. However, the main circulating form of vitamin K is phylloquinone whereas the common hepatic forms MK-9 to MK-13 are not detectable in the plasma (Shearer et al., 1996). Unlike other fat-soluble vitamins, bodily stores of vitamin K are rapidly depleted (Suttie et al., 1988); it seems that 60-70% of absorbed phylloquinone is ultimately lost by excretion in the urine (20%) and in the faeces (40-50%) (Shearer et al., 1974; Usui et al., 1990).

The activity of various vitamin K forms is the sum of the relative absorption, transport, metabolism and effectiveness of this compound in normal vitamin K-dependent functions. Differences in the activity have been studied in a few studies; most of them (Will and Suttie, 1992; Groenen-van Dooren et al., 1993; Reedstrom and Suttie, 1995) have shown phylloquinone to be more effective than MK-4 or MK-9 in maintaining normal vitamin K

status. However, Groenen-van Dooren et al. (1995) observed later that MK-9 has a longer effect than phylloquinone or MK-4 on prothrombin synthesis.

2.2.2 Function

Until to mid-1970s it was believed that the physiologic role of vitamin K is limited to the synthesis of clotting factors (prothrombin and factors VII, IX, and X). After the discovery of Gla (Nelsestuen and Zytkovicz, 1974; Stenflo et al., 1974), it was shown that vitamin K acts as a cofactor in the posttranslational synthesis of Gla from glutamic (Glu) residues. This led to the identification of additional vitamin K-dependent proteins also including extrahepatic proteins with no connection to blood coagulation. Isolation of these proteins from bone (osteocalcin, matrix Gla protein and protein S) expanded the physiological role of vitamin K significantly (Suttie, 1992; Ferland, 1998; Vermeer et al., 1998).

The role of vitamin K in the posttranslational conversion of protein bound glutamate to Gla residues, which are common to all vitamin K-dependent proteins and increase their affinity to calcium, is illustrated in Figure 2. This γ-carboxylation is catalysed by vitamin K-dependent carboxylase, which requires the reduced form of vitamin K, hydroquinone, and the energy provided by simultaneous oxidation of hydroquinone to vitamin K 2,3-epoxide. This form is then recycled to its quinone and further to its hydroquinone form in reactions catalysed by vitamin K reductases. Vitamin K deficiency leads to the synthesis of undercarboxylated proteins, which are secreted in the plasma. These proteins called PIVKAs (protein induced by vitamin K absence) have low affinity for calcium and have been used as new markers of suboptimal vitamin K nutrition (Ferland, 1998; Vermeer et al., 1998).

After the finding of vitamin K-dependent proteins in bone, the role of vitamin K in calcium homeostasis has been under active research. Despite this the precise functions of these proteins in bone metabolism are still unknown; it is, however, assumed that at least osteocalcin and matrix Gla protein have a regulatory function in the formation of the bone mineral matrix and in maintenance of healthy mature bone (Shearer, 1995; Vermeer et al., 1998). For examples Hodges et al. (1991, 1993) have reported low circulating phylloquinone and menaquinone levels to be associated with osteoporotic hip fractures. As reviewed by Vermeer et al. (1998) this led to further studies in which undercarboxylated osteocalcin (ucOC) has been found to be a sensitive marker of hip fracture risk (e.g. Szulc et al., 1993, 1994; Sokoll et al., 1997). In addition, both Feskanich et al. (1999) and Booth et al. (2000a) ob-

served that low dietary vitamin K intake significantly increases the risk of hip fracture. On the other hand, also conflicting results are reported, for example no association has been found between vitamin K intake and bone mineral density (Rosen et al., 1993; Booth et al., 2000a). Although the current data suggested an association between vitamin K deficiency and the development of osteoporosis, evidence of the beneficial effects of vitamin K is not as compelling as in the case of vitamin D (Binkley and Suttie, 1995; Shearer, 1997).

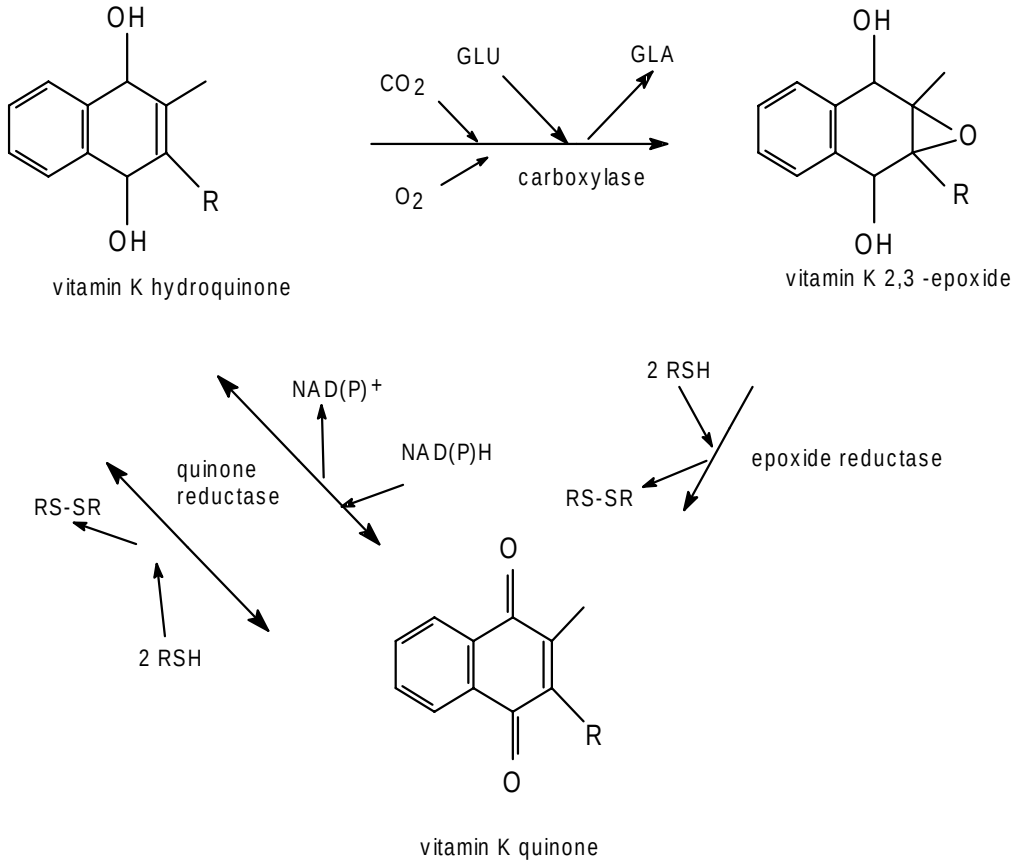

Figure 2. The vitamin K cycle. R= the side chain of K vitamer (Jukes, 1988).

2.2.3 Deficiency

A primary deficiency of vitamin K is uncommon in healthy humans because of wide distribution of phylloquinone among green vegetables and synthesis of menaquinones by the intestinal microflora. Vitamin K deficiency has been reported only in special cases, for example in patients with low dietary intakes who are also receiving antibiotics (Suttie, 1992). The risk of serious vitamin K deficiency is greatest among newborn infants, because their

vitamin K stores are low, their sterile guts do not produce menaquinones, and vitamin K content in human milk is low. This deficiency is called a hemorrhagic disease of the newborn, and it is prevented in many countries by prophylaxis of phylloquinone at birth (Suttie, 1992; Shearer, 1995).

New findings about vitamin K function in bone metabolism have, however, raised the question of whether vitamin K deficiency is more common (Vermeer et al., 1998). It is unclear, what are the best markers to evaluate vitamin K status. The previously used prothrombin time does not react before deficiency is very serious, but for examples the amount of PIVKAs in serum increases much earlier (Suttie, 1992; Booth and Suttie, 1998). In addition, Suttie et al. (1988) observed that 50-100 μg of phylloquinone is needed per day to maintain the ratio of undercarboxylated and active prothrombin near to normal value; however, a bigger dietary intake is needed before serum phylloquinone concentration is restored after vitamin K depletion. The current recommended dietary vitamin K intakes; Recommended Dietary Allowance (RDA) in USA and Dietary Reference Value (DRV) in UK, are based only on the role of vitamin K in blood coagulation. According to them a sufficient dietary intake is 1 μg/kg (body weight) in day (Olson, 1987; Food and Nutrition Board, 1989). Before new recommendations can be given, more research about the significance of vitamin K on bone metabolism is needed. Also the bioavailability of different vitamin K forms and dietary sources as well as of menaquinones synthesised by the intestinal microflora has to be clarified.

2.3 Determination in foods

Biological, chemical and chromatographic methods have been used to determine vitamin K compounds in food; only HPLC methods are nowadays regarded as reliable (Booth et al., 1993). There is no official method for the measurement of vitamin K in foods generally, except for the method for determining vitamin K in milk and infant formulas (Indyk and Woollard, 1997, 2000; AOAC, 2000). In this method the phylloquinone content is determined with HPLC by fluorescence detection after lipase treatment and *n*-hexane extraction. Upgrading the status of this method to an Official AOAC one, it is also being undertaken by CEN (European Committee for Standardization). Due to the complexity of the matrices and the low vitamin K contents in foods the methods used must be of high sensitivity and

selectivity. Although HPLC with different extraction and purification procedures has greater potential to determine vitamin K in foods than other methods, the detection of vitamin K compounds has remained an analytical challenge, especially in the case of menaquinones (Lambert and de Leenher, 1992). Because of the sensitivity of vitamin K to daylight, sample preparation and analysis should be done under subdued light regardless of the method. In addition, the sensitivity of the vitamin to strong alkaline decreases the number of possible methods, which can be used in the isolation step (Fauler et al., 2000).

2.3.1 Biological methods

Until the beginning of 1980s all methods suitable for determining vitamin K in foods were bioassays, in which chicks were usually used as test animal as reviewed by Parrish (1980). Both a preventive test, in which prothrombin times between animals receiving known amounts of vitamin K and animals receiving a vitamin K-free diet were compared, and a curative test have been used. Although a standard bioassay method has never been developed, the curative test is though to be the best technique. In this test chicks first receive a vitamin K-free diet to deplete their body stores. The chicks are then divided into groups and fed with diets supplemented with a graded series of known amounts of vitamin K to establish a response curve or with a test sample with unknown vitamin K content. After short (18- 24 h) or long (two weeks) test periods, either the clotting times or prothrombin times of the animals' whole blood are compared (Parrish, 1980; Suttie, 1984).

Numerous variables affect the results in the chick bioassay, such as test animal, differences in food intakes between test groups, the length of the test period and the standard used for comparison. Thus the reported vitamin K values are in wide range. Bioassays have today mainly been replaced with chromatographic methods. They are, however, still needed for establishing the biological activity of different K vitamers and to determine the bioavailability of vitamin K in foods (Parrish, 1980).

2.3.2 Chemical methods

The most widely used chemical methods for determining vitamin K are colorimetric tests. They are not specific for vitamin K as other quinones also react in a similar manner. According to the review of Parrish (1980) the reaction with xanthine hydride in ethanolic KOH solution produces a stable orange colour, which is quite specific for vitamin K. Other

colorimetric tests, such as ceric sulphate titration, have also been used although they are not very sensitive (Parrish, 1980).

2.3.3 Gas-liquid chromatographic methods (GC)

The possibilities of using GC in vitamin K analysis were extensively explored during the 1960s and 70s but this method has never become very popular. GC methods have been reviewed by Sheppard et al. (1972), Parrish (1980) and Lambert and de Leenher (1992). In most of the published studies only synthetic K vitamers have been analysed; for example menadione (a synthetic vitamin K form) has been determined successfully by GC (Sheppard and Hubbard, 1971; Parrish, 1980). The greatest reasons for the unpopularity of GC in vitamin K analysis are long retention times and possible degradation of compounds on the column because high temperatures (300 °C) are needed due to the low volatility of K vitamers (Lambert and de Leenher, 1992). Despite these problems Seifert (1979) has developed a GC method to analyse phylloquinone in green vegetables after *n*-hexane extraction and purification on alumina columns. He found the method to be relatively simple and feasible; no derivatives were needed and no thermal breakdown occurred. However, HPLC methods have replaced GC methods in vitamin K analysis. On the other hand, after the introduction of new technologies, such as GC-MS and special fused silica columns, determination of vitamin K with GC have again become objects of interest (Fauler et al., 2000); for example Davidson et al. (1996) applied GC-MS to confirm the identification of dihydrophylloquinone in hydrogenated oils.

2.3.4 High-performance liquid chromatographic methods (HPLC)

Nowadays, high-performance liquid chromatography is the most popular method for the routine analysis of vitamin K in foods. Its introduction facilitated analysis, for example, by decreasing analytical variation as well as increasing sensitivity and resolution (Lambert and de Leenher, 1992; Booth et al., 1993). Other advantages of HPLC are the absence of the risk for thermal degradation, protection against light during the chromatographic run and its versatility because various stationary phases and detection systems can be used with it. There are problems in vitamin K analysis also with HPLC; despite preliminary purification of food extracts many compounds can interfere the analysis, identification of menaquinones is uncertain and its sensitivity is still inadequate for foods with very low vitamin K contents (Lambert and de Leenher, 1992; Fauler et al., 2000). Several HPLC methods for determin-

ing the presence of naturally occurring K vitamers, especially for phylloquinone, have been developed, more recent examples of which are given in Table 1.

Extraction procedures

Due to instability of K vitamers in alkali conditions, saponification, which is generally used in extraction of fat-soluble vitamins, is not suitable for vitamin K analysis. Thus vitamin K compounds are usually extracted from foods with common lipid extraction methods. The choice of extraction solvents depends on the food matrix; normally vitamin K is extracted with nonpolar fat solvents but sometimes more polar solvents are used. It has been suggested that phylloquinone is tightly bound to membranes, so, vortexing with some mechanical apparatus or sonification are often used to achieve more efficient extraction.

In recent studies the use of the mixture of 2-propanol and *n*-hexane as an extraction solvent for various food matrices has been the most often reported method (Hirauchi et al., 1989a; Langenberg et al., 1986; Ferland and Sadowski, 1992a; Booth et al., 1994, 1995, 1996b; Jakob and Elmadfa, 1996). In the case of vegetables and cereal products also the mixtures of dichloromethane and methanol (Jakob and Elmadfa, 1996) and acetone and *n*-hexane (Gijsbers et al., 1996) have been used whereas the use of *n*-hexane with methanol, 2-propanol, acetone or ethanol has been reported for the extraction of various animal products (Fournier et al., 1987; Hirauchi et al., 1989b; Udagawa et al., 1993; Schurgers et al., 1999). Although it is generally assumed that saponification is not suitable for vitamin K, Careri et al. (1996) reported successful (recovery 82%) use of mild alkali hydrolysis. They extracted phylloquinone from vegetables with *n*-hexane after heating for one hour in sodium carbonate solution.

Table 1. Some recent HPLC methods used for the determination of vitamin K in foods

Food	Vitamin K compounds (internal standard)	Column	Mobile phase	Detection	Reference
Oils	Phylloquinone	Supelco C_{18} (150 x 4.6 mm, 5 μm)	Methanol-acetonitrile-water (88:10:2)	UV 270 nm	Zonta and Stancher, 1985
Milk and infant formulas	Phylloquinone (cholesteryl phenylacetate)	Resolve C_{18} (100 x 8 mm, 5 μm)	Methanol-2-propanol-ethyl acetate-water (450:350:145:135)	UV 269 nm and 277 nm	Indyk et al., 1995
Vegetables	Phylloquinone	LiChrosorb RP-8 (250 x 4.6 mm, 10 μm)	Methanol	UV 247 nm + MS	Careri et al., 1996
Human milk	Phylloquinone (MK-7)	Radial Pak C18 (10 μm)	Ethanol-hexane-water (90:6,5:3,5) + 25 mM TBAP	EC -0.6V / +0.2 V	Canfield et al., 1990, 1991
Human milk	Phylloquinone	OD-224, RP18 (220 x 4.6 mm, 5 μm)	Methanol:water (99:1) +2.5 mM CH_3COOH / NaAc	EC -1.2 V / +1.5 V	Zamarreno et al., 1995
Plant foods	Phylloquinone (MK-6)	Hypersil MOS (5 μm)	Methanol-water (92.5:7.5) + 30 mM $NaClO_4$	FL 320 / 430 nm Electrochemical Reduction	Langenberg et al., 1986
Animal tissue	A: phylloquinone, MK-4 to MK-9 B: MK-10 to MK-14	Nucleosil C_{18} (150 x 4.6 mm, 5μm)	0.25% $NaClO_4$ in A: 92.5% ethanol or in B: 97.5% ethanol	FL 320 / 430 nm Electrochemical reduction	Hirauchi et al., 1989a

Oils	Phylloquinone ($K_{1(25)}$)	Novapak C18 (150 x 4.6 mm, 4 µm)	Acetonitrile-ethanol (95:5) + 5 mM $NaClO_4$	FL 320 / 430 nm Electrochemical reduction	Moussa et al., 1989, 1994
Various foods	Phylloquinone and MK-4 to MK-10 (MK-3)	Nucleosil C_{18} (250 x 4.6 mm, 5 µm)	Methanol-ethanol-water (1:2:0.06)	FL 254 / 430 nm Reduction with PtO_2 (reactor 50 x 4.6 mm)	Shino, 1988
Oils and vegetables	Phylloquinone (Dihydrovitamin K_1)	Hypersil ODS (250 x 4.6 mm, 5µm)	Dichloromethane-methanol (20:80) + 10 mM $ZnCl_2$, 5 mM CH_3COOH and 5mM NaAc	FL 248 / 418 nm Reduction with Zn (reactor 20 x 3.9 mm)	Ferland and Sadowski, 1992a, b (Haroon et al., 1986)
Various foods	Phylloquinone and dihydrovitamin K_1 ($K_{1(25)}$)	Hypersil ODS (150 x 4.6 mm, 3µm)	Dichloromethane-methanol (10:90) + 10 mM $ZnCl_2$, 5 mM CH_3COOH and 5mM NaAc	FL 244 / 418 nm Reduction with Zn (reactor 50 x 2.0 mm)	Booth et al., 1994, 1995, 1996b; Davidson et al., 1996
Oils	Phylloquinone (MK-4)	PartiSphere C_{18} (150 x 4.6 mm, 5 µm)	Methanol-acetonitrile (85:15) + 10 mM $ZnCl_2$, 5 mM CH_3COOH and 5mM NaAc	FL 254 / 400 nm Reduction with Zn (reactor 150 x 3.9 mm)	Gao and Ackman, 1995
Milk and infant formulas	Phylloquinone and menaquinone-4	Resolve C_{18} (100 x 8 mm, 5 µm)	Dichloromethane-methanol (10:90) + 10 mM $ZnCl_2$, 5 mM CH_3COOH and 5mM NaAc	FL 243 / 430 nm Reduction with Zn (reactor 20 x 4.0 mm)	Indyk and Woollard, 1997, 2000; AOAC, 2000
Margarines	Phylloquinone and dihydrovitamin K_1	YMC C_{30} (250 x 4.6 mm, 3µm)	Dichloromethane-methanol (10:90) + 10 mM $ZnCl_2$, 5 mM CH_3COOH and 5mM NaAc	FL 243 / 430 nm Reduction with Zn (reactor 20 x 4.0 mm)	Cook et al., 1999

The published extraction procedures are more similar when analysing the vitamin K contents of liquid samples. *n*-Hexane is the most common solvent used to extract phylloquinone from oils and margarines (Ferland and Sadowski 1992b; Moussa et al., 1994; Gao and Ackman, 1995; Davidson et al., 1996; Jakob and Elmadfa, 1996; Cook et al., 1999) although also extraction with pentane or mixture of 2-propanol and *n*-hexane has been reported (Zonta and Stancher, 1985; Booth et al., 1994, 1996b). In the case of milk and infant formula samples, vitamin K compounds are usually extracted after lipase treatment either with *n*-hexane (Indyk et al., 1995; Zamarreno et al., 1995; Indyk and Woollard; 1997, 2000) or with pentane (Barnett et al., 1980; Bueno and Villalobos, 1983; Isshiki et al., 1988). The use of dichloromethane with 2-propanol (Landen et al., 1989), methanol (Haroon et al., 1982) or iso-octane (Hwang, 1985; Tanner et al., 1993) as well as a mixture of 2-propanol and *n*-hexane (Canfield et al., 1990) are exceptions in the extraction procedures used for this food group.

Recently also new extraction techniques have been applied in vitamin K analysis. Chase et al. (1999, 2000a,b) have used matrix solid-phase dispersion (MSPD) to extract phylloquinone from infant formulas and various medical foods. In this method C18 is used as extraction phase and 0.5% 2-propanol in hexane and ethyl acetate are used as extraction solvents. Isopropyl palmitate is needed as a keeper solvent for the phylloquinone. Chase et al. (1999, 2000a,b) found MSPD to be a simple and rapid technique, in which both solvent and sample amounts needed are small, to analyse phylloquinone in these quite homogenous matrices. Later they coupled MSPD with accelerated solvent extraction (ASE) successfully to analyse phylloquinone from medical foods. The coupling of these two techniques both automated their use and broadened their applicability to solid matrices (Chase and Thompson, 2000). However, it is not known if these methods can be used for ordinary food items.

In most of the published studies the extraction procedures are inadequately documentated. Although it is generally assumed that in foods at least phylloquinone is tightly bound to membranes, special attention is rarely focused on this. Moreover, only very rarely are descriptions of the optimisation of other steps in extraction given. In addition, most of recent studies have concentrated on phylloquinone and plant foods thus extraction procedures particularly for menaquinones and animal products have not been developed. In conclusion, the extraction procedures have to be tested and validated carefully before reliable vitamin K data can be produced.

Cleanup

Crude sample extracts cannot usually be used directly in HPLC analysis because phylloquinone and menaquinones generally occur at lower concentrations than other lipophilic compounds in foods. In addition to eliminate interfering compounds, extensive purification is done to extend column life by removing late-eluting compounds (Gao and Ackman, 1995). Various methods including lipase treatment, solid-phase extraction (SPE) and semipreparative HPLC have been used for purification. Usually two different purification methods are needed; the combination of normal and reverse-phase chromatography is generally regarded as the most effective method to resolve vitamin K from contaminating lipids (Canfield and Hopkinson, 1989; Canfield et al., 1990). The food matrix and vitamin K forms as well as the analytical system, however, influence the selection of the purification method. For example, in some studies analysing oils and vegetables extraction with organic solvents was shown to be able to purify extracts sufficiently by removing water-soluble contaminants (Langenberg et al., 1986; Moussa et al., 1994; Gijsbers et al., 1996; Jakob and Elmadfa, 1996; Cook et al., 1999; Schurgers et al., 1999). In addition, Chase et al., (1999, 2000a,b) have analysed phylloquinone contents of infant formulas and medical foods directly after MSPD extraction.

Lipase treatment. When analysing phylloquinone content in oils, milk and infant formulas enzymatic hydrolysis of triglycerides before extraction has been used quite often either alone (Barnett et al., 1980; Bueno and Villalobos, 1983; Indyk and Woollard, 1997, 2000; Cook et al., 1999) or with another purification method (Zonta and Stancher, 1985; Isshiki et al., 1988; Lambert et al., 1992; Gao and Ackman, 1995; Indyk et al., 1995; Zamarreno et al., 1995). Generally, lipase powder and a buffer (for example phosphate buffer) are added to liquid sample, which is then incubated at 37 °C for 90-120 min. After this vitamin K is extracted with *n*-hexane or pentane. According to Lambert et al. (1992) disturbing the fat globules, for example by sonication, before lipase treatment is necessary for optimal removing of fat from milk. However, in several studies only magnetic stirring during incubation is used.

Open-column chromatography. In addition to lipase treatment, open-column chromatography is another generally used purification method when analysing the phylloquinone contents of milk and infant formulas. Silica is the most commonly used column material (Haroon et al., 1982; Hwang, 1985; Shino, 1988; Landen et al., 1989; Canfield et al., 1990; Tanner et al., 1993) although purifications have also been made with alumina (Manes et al., 1972; Zonta and Stancher, 1985; Shino, 1988). Among the published studies columns of various size (5-20

g) as well as different organic solvents have been applied. Although this technique effectively removes both less polar and more polar lipids than phylloquinone, many lipids are still present in the extracts. Thus open-column chromatography is often used together with another purification method (Lambert and de Leenher, 1992).

Solid-phase extraction (SPE). Nowadays, straight-phase SPE is the most common purification method when analysing phylloquinone and menaquinones in various food matrices (Hirauchi et al., 1989a,b; Ferland and Sadowski, 1992a,b; Udagawa et al., 1993; Booth et al., 1994, 1995, 1996b; Gao and Ackman, 1995; Zamarreno et al., 1995; Davidson et al., 1996). SPE is regarded as a method, which readily separates vitamin K from the coextractable compounds found in food samples. It has replaced open-column chromatography due its rapidity although its resolving power is sufficient only for food items with low fat content (Canfield et al., 1990). When using silica cartridges in purification, nonpolar compounds are removed from cartridge with *n*-hexane; after washing K vitamers are eluted with a slightly more polar solvent. Food items with a high fat content (for example meats, butter and margarine) have been purified further by various methods including reverse-phase SPE (Booth et al., 1994, 1995, 1996b; Davidson et al., 1996). When using C18 cartridges a mixture of methanol and water together with acetonitrile are generally used for washing, after which K vitamers are eluted with a mixture of methanol and dichloromethane.

Semipreparative HPLC. Semipreparative straight-phase HPLC has been used as the second purification method after lipase treatment or open-column chromatography in a few studies analysing vitamin K from milk and infant formulas (Haroon et al., 1982; Fournier et al., 1987; Indyk et al., 1995). It is a practical method, which removes different classes of lipids from sample extracts (Lambert and de Leenher, 1992). In this method the fraction containing K vitamers is isolated on the HPLC column, detected with UV and collected; the concentrated fraction is then used for analysis. Silica columns have been most frequently used due to high lipid content of many sample extracts, although also the use of amino (Landen et al., 1989) and amino-cyano (Haroon et al., 1982) columns have been reported. In addition, Isshiki et al. (1988) and Canfield et al. (1990) have used reverse-phase semipreparative HPLC. In these exceptions, however, sample extracts were already purified with silica using different techniques (Haroon et al., 1982; Landen et al., 1989; Canfield et al., 1990) or with lipase (Isshiki et al., 1988). The advantage of straight-phase chromatography as a purification method is that

various vitamin K forms elute quite close to each other so a narrow fraction can be collected and a lot of interfering compounds eliminated (Lambert and de Leenher, 1992).

Summary of cleanup procedures. In addition to the earlier mentioned methods, liquid-phase reductive extraction purification (Booth et al., 1994, 1995) and TLC with silica gel plates (Hirauchi et al., 1989a,b) have been used for purification of vitamin K extracts. In summary no universal purification method for vitamin K analysis exist. The separation of K vitamers from interfering compounds is typically based on absorption chromatography although the techniques used vary. Among the published studies various purification methods have been combined in many ways; chromatographic purification after lipase treatment is the most common. The suitability of the purification methods is often tested only for phylloquinone analysis. The large polarity scale of menaquinones has to be remembered when selecting a purification method for them. On the other hand, the food matrix (especially fat content) makes its own demands on the method. Thus, it is probably not possible to use only one purification method in all vitamin K analysis. However, more research is needed to evaluate the advantages and disadvantages of various cleanup methods for different food matrices.

Quantification

Due to chemical and physical properties of K vitamers, the final analysis can be performed in numerous ways. However, reverse-phase chromatography is the most commonly used technique because it provides good separation of K vitamers from interfering compounds after using straight-phase chromatography generally for cleanup (Canfield and Hopkinson, 1989; Fauler et al., 2000). Although various packing materials are available, the C18 column is the most common. There are, however, few exceptions among the published studies; Cook et al. (1999) used the C30 column in margarine analysis and Udagawa et al. (1993) used the C8 column in fish analysis. In addition, Hwang (1985) analysed *trans* and *cis*-isomers of phylloquinone in infant formulas with straight-phase system. These isomers can also be separated with the C30 column (Cook et al., 1999) but they coelute with C8 and C18 columns. Because only the *trans* form of phylloquinone exists naturally, it is not usually thought that their separation is necessary. Phylloquinone in milk and infant formulas has also been analysed together with other fat-soluble vitamins using C18 columns for separation (Barnett et al., 1980; Landen et al., 1989; Zamarreno et al., 1995).

When methods are very similar in regard to the stationary phase, large variations exist in the mobile phase applied for vitamin K analysis (Table 1). The detection system affects the selection of a mobile phase to a high degree. In addition, analysing both phylloquinone and menaquinones requires a different mobile phase than that used when analysing only phylloquinone. Moreover, gradient elution is usually needed for the efficient separation of menaquinones, whereas isocratic elution is suitable for phylloquinone (Canfield and Hopkinson, 1989; Lambert and Leenher, 1992; Eitenmiller and Landen, 1999; Fauler et al., 2000).

A variety of detection methods have been applied when analysing vitamin K from food items with HPLC as summarised in Table 1. Detection of phylloquinone and menaquinones is possible with UV, electrochemical (EC), fluorescence (FL) after reduction to the corresponding hydroquinones, and mass selective (MS) detectors (Lambert and de Leenher, 1992; Fauler et al., 2000). The FL detection after chemical reduction is the most popular system although UV detection is used quite often in milk and infant formula analysis.

UV detection. Vitamin K displays a relatively poor UV absorbance having four peaks in its UV spectra; absorbance is the highest at 248 nm, which is, however, a rather nonselective wavelength. Thus other wavelengths, such as 254 nm and 270 nm or both wavelengths together, are generally used to obtain better selectivity (Fauler et al., 2000). According to Indyk et al. (1995) the detection limit of UV detector is 1 ng per injection; this is hardly sufficient to quantify natural vitamin K contents in food samples. Despite this it has been used commonly when analysing infant formulas, to which synthetic phylloquinone has been added (Barnett et al., 1980; Haroon et al., 1982; Bueno and Villalobos, 1983; Hwang, 1985; Landen et al., 1989; Tanner et al., 1993; Indyk et al., 1995) and also in a few other studies (Zonta and Stancher, 1985; Fournier et al., 1987; Careri et al., 1996).

Electrochemical detection. Although electrochemical detection (EC) is regarded as a simple and reproducible detection method for vitamin K analysis (Canfield and Hopkinson, 1989), it has not become very popular in food analysis. In its first applications quinone forms of vitamin K were reduced to hydroquinones by applying a negative potential to electrode; this resulted in a current proportional to the amount of vitamin K reduced. This system is very sensitive to oxygen in mobile phase resulting in a high background current. In addition, its sensitivity is decreased easily due to passivation of the working electrode (Lambert and de Leenher, 1992).

Due to these problems with reductive electrochemical detection, the dual-electrode detection system (also called redox mode) was developed. In this technique, which was first applied for plasma and rat liver (Haroon et al., 1984; Hart et al., 1985), quinone forms of vitamin K are first reduced to hydroquinone forms by negative potential of the first (upstream) electrode. These hydroquinone forms are immediately oxidised back to quinones on the second (downstream) electrode and the resulting current is determined. Because this reoxidation is reversible for vitamin K, detection can be done at lower positive potential than in reductive EC detection. Thus better chromatograms are achieved due to smaller effect of baseline drift.

The main advantage of a dual-electrode system is that it eliminates interfering oxygen. In addition, the selectivity can be increased further by applying a guard cell to the system directly after the pump to remove interfering metal ions by oxidising them. The sensitivity of dual-electrode EC detection is superior when compared to UV; Hart et al. (1985) reported the detection limit for phylloquinone standard to be 50 pg per injection. In addition, Haroon et al. (1984) suggested that this technique would be sensitive enough also to menaquinone analysis. Despite the use of the dual-electrode system the sensitivity of detector can be reduced to an unacceptable level quite easily by the absorption of coeluted compounds on the surfaces of the electrodes. This can be prevented by recycling mobile phase or reversing the potentials between analysis series (Hart et al., 1985). Another disadvantage is that the sample has to be dissolved in semi-aqueous solvent, in which also the required electrolyte (for example NaAc or perchlorate) can be dissolved. The dual-electrode EC detection has been applied only in a few studies analysing vitamin K in foods (Isshiki et al., 1988; Canfield et al., 1990; Zamarreno et al., 1995).

Fluorescence detection. In most of the recent studies vitamin K is detected with fluorescence detection (FL), which provides much better sensitivity and selectivity than can be achieved with UV detection. Because vitamin K compounds do not exhibit natural fluorescence, several methods have been developed to produce the corresponding fluorescing hydroquinones. These reduction methods include electrochemical and various chemical methods (Fauler et al., 2000).

In the first fluorescence applications quinone forms of vitamin K were reduced electrochemically. According to Langenberg and Tjaden (1984a,b) the absence of oxygen is essential for the detection. They also optimised other conditions of their detection system, in which EC

detector was used as a post-column reactor. They observed that the effect of flow-rate of mobile phase or concentration of electrolyte to the fluorescence is minor, instead the composition of mobile-phase influences both selectivity and sensitivity. They could achieve a detection limit as low as 25 pg per injection. Later it was observed that this detection system is not very reproducible; Moussa et al. (1989) suggested that the passivation of the electrodes is the reason for this. Generally the advantages and disadvantages of this method are similar with the EC detection, for example when selecting potentials a balance between sensitivity and background noise have to be found. Both phylloquinone and menaquinone contents of various food items have been detected with fluorescence after electrochemical reduction (Langenberg et al., 1986; Hirauchi et al., 1989a,b; Moussa et al., 1994, Schurgers et al., 1999). The wavelengths and mobile-phases used are summarised in Table 1.

The chemical reduction of K vitamers to corresponding hydroquinones can be done in many ways. The first applications were wet-chemical reduction systems with various reagents. These systems, however, have quite many problems, such as long elution times, a complicated technique and high reaction temperatures. Thus wet-chemical reduction has never been applied for food analysis, although some modifications for plasma analysis exist (Lambert et al., 1986; Fauler et al., 2000).

Post-column reduction on a solid-phase reactor is the more often used chemical reduction system, in which both platinum oxide and zinc have been used as catalysts for reduction (Eitenmiller and Landen, 1999; Fauler et al., 2000). A fluorimetric method, in which the reduction of K vitamers take place on postcolumn reactor packed with zinc particles, was introduced in the 80s (Haroon et al., 1986, 1987). $ZnCl_2$, NaAc and acetic acid have to be added to the mobile phase, which is usually 10-20% dichloromethane in methanol, to catalyse the reaction. Haroon et al. (1987) found this method to have many advantages over electrochemical reduction; for example non-aqueous mobile phase is more suitable for the separation of K vitamers and the reactor is not passivated very easily. According to their results the reduction efficiency of the method was 95% and the detection limit for phylloquinone 25 pg and for menaquinones 100 pg (per injection). This method is regarded as a highly specific assay for vitamin K (Eitenmiller and Landen, 1999) and has been applied in many studies analysing vitamin K in various food items (Ferland and Sadowski, 1992a,b; Booth et al., 1994, 1995, 1996b; Gao and Ackman, 1995; Davidson et al., 1996; Jakob and Elmadfa, 1997; Indyk and Woollard, 1997, 2000; Cook et al., 1999). Although the principles of the method are same in

all studies, small modifications in wavelengths, mobile phases and sizes of the zinc column exist (Table 1).

In other solid-phase reactor applications platinum oxide has been used as a catalyst for reduction of quinones to hydroquinones. This reduction system does not need any reagents in the mobile phase. According to Iwase (2000) this method is simple, rapid, highly selective and reproducible; reported detection limits (per injection) were 25 pg for phylloquinone and 25-150 pg for various menaquinones (Shino, 1988). Platinum oxide catalyst chemical reduction have been used in a few studies analysing vitamin K in animal foods (Shino, 1988; Udagawa et al., 1993).

Fluorescent hydroquinones can also be formed by photochemical decomposition although no applications for food analysis have been published (Fauler et al., 2000). The method is based to photodegradation of K vitamers after irradiation by strong light. One of the reaction products is fluorescent hydroquinone. Lefevere et al. (1982) reported a detection limit of 150 pg per injection for phylloquinone by this method. The selectivity of this method is good, and no reagents are needed for reduction (Lefevere et al., 1982). Later Indyk (1988b) developed even more simple method, in which photochemical reduction occurred in the flow cell of a fluorescence detector. The sensitivity of this method was, however, very poor (detection limit 10 ng per injection).

Mass selective detection (MS). Although new improvements in interfaces have made the use of LC-MS in routine analysis possible, this technique has been applied only seldom in vitamin K analysis (Fauler et al., 2000). Sakano et al. (1986) and Sano et al. (1997) have used MS to confirm the identification of menaquinones in physiological samples. Careri et al. (1996) used particle beam LC-MS for the detection and unambiguous characterisation of phylloquinone in vegetable samples. They found the detection limit of this technique in single-ion monitoring mode to be 2 ng per injection. Their results determined both with MS and UV detectors were comparable to those in the literature; in the case of a tomato sample, however, there was an overestimation in the UV detection because of the coelution of lycopene with phylloquinone. Thus, the use of LC-MS to confirm UV data was observed to be necessary. Despite the few published studies, LC-MS seems to be a promising technique at least for qualitative analysis of vitamin K (Fauler et al., 2000).

The summary of quantification methods. The quantification methods are mainly developed for analysing phylloquinone in plasma. Menaquinones have been determined only in a few studies; their separation and detection is still a great challenge. The validation of the methods for food matrices is usually inadequate; for example the authenticity of vitamin K peaks is confirmed only in a few studies either by changing the wavelengths of fluorescence detector (Booth et al., 1994; Gao and Ackman, 1995; Jakob and Elmadfa, 1996) or by removing the reduction reactor (Ferland and Sadowski, 1992a). It is probable that the unambiguous identification of vitamin K needs the use of two different detection systems as has been shown by Careri et al. (1996).

The summary of HPLC methods. Because of the many steps in sample preparation and derivatization in quantification, the use of an internal standard is regarded to be necessary (Haroon et al., 1987; Booth et al., 1993). However, finding an universal internal standard suitable for all vitamin K analysis is difficult. Therefore many alternatives, such as $K_{1(25)}$, MK-4 and dihydrovitamin K_1, have been applied with good and not so good success rates (Booth and Sadowski, 1997). As already mentioned there are also defects in documentation and validation of the other steps of analysis. Reliable data, however, can only be obtained if systematic quality control procedures, which consider every step in the method, are instituted and routinely monitored. Although present methods and detection techniques create a good foundation for vitamin K analysis, further improvements in different steps of analysis are needed before both phylloquinone and menaquinones can de determined from various food matrices reliably.

2.4 Vitamin K in foods

Vitamin K exists naturally in two forms, as phylloquinone and menaquinones. Phylloquinone has a ubiquitous distribution in the diet, the range of concentrations in different food categories is very wide (Shearer et al., 1996). Generally dark-green, leafy vegetables are regarded as the best dietary sources (Booth et al., 1996a; Booth and Suttie, 1998). The occurrence of bacterially synthesised menaquinones in foods is quite unknown; the main dietary sources of menaquinones seem to be cheeses and fermented soybean products (Shearer et al., 1996).

The knowledge of vitamin K contents in foods is limited although database for phylloquinone has been grown significantly over the past few years (Suttie 1992; Booth et al., 1993; Shearer et al., 1996). Most food composition tables either do not include values for vitamin K, or the values cited are of questionable worth because they are from studies, in which the validity of extraction and analytical methods is insufficiently validated (Booth et al., 1993). Some figures are still derived from bioassays intended only as a qualitative guide to vitamin K content (Suttie 1992). In addition, the aim of many studies has been mainly method development, and the representativenes of the sampling and its documentation have received minor attention. Thus, more detailed information on vitamin K in foods is needed, especially the distribution of menaquinones and their contents in foods of animal origin have to be determined. In the case of phylloquinone the variation occurring in the contents of various foods is worth of investigations. Only HPLC results have been included in the following summarisation.

2.4.1 Foods of plant origin

Most of the published vitamin K studies have concentrated on plant foods, which naturally only contain phylloquinone. On the other hand, during food processing also other vitamin K compounds can be formed including dihydrovitamin K_1 in hydrogenated oils (Davidson et al., 1996) and menaquinones in fermented soybean products (Shino, 1988).

Phylloquinone levels among various vegetables differ from extremely low to considerable high; it is generally assumed that phylloquinone content correlates with the strength of green colour in these items. The highest amounts (over 300 μg/100 g) have been found in dark-green, leafy vegetables, for example spinach and kale (Langenberg et al., 1986; Shino, 1988; Ferland and Sadowski, 1992a; Weihrauch and Chatra, 1993; Booth et al., 1994, 1995; Careri et al., 1996; Shearer et al., 1996). Phylloquinone levels are also high among other green vegetables; typical contents reported for various lettuces and cabbages are 50-200 μg/100 g, whereas yellow and red vegetables as well as root crops contain phylloquinone only in low amounts (1-25 μg/100 g) (Langenberg et al., 1986; Ferland and Sadowski, 1992a; Weihrauch and Chatra, 1993; Booth et al., 1994; Careri et al., 1996; Jakob and Elmadfa, 1996; Shearer et al., 1996). The situation is the same for fruits and berries, in which the reported phylloquinone amounts are usually under 5 μg/100 g; only green fruits, such as avocado, contain more phylloquinone (even 40 μg/100 g) (Weihrauch and Chatra, 1993; Booth et al., 1995; Shearer et al., 1996). Most cereal products contain less than 5 μg phylloquinone per 100 g, except

bakery products with a high fat content, for example crackers and muffins (13-25 μg/100 g) (Weihrauch and Chatra, 1993; Booth et al., 1994, 1995; Shearer et al., 1996).

There is great variation in figures reported for vegetables; it is not known if variation is biological or analytical in origin (Booth and Sadowski, 1997). Ferland and Sadowski (1992a) compared phylloquinone contents of five different vegetables grown at two different locations and observed significant differences. For example in the case of kale the range was from 621 μg/100g to 1657 μg/100g. They suggested that possible reasons for variations are differences in climate, soil and growing conditions.

In addition to green vegetables, plant oils are significant dietary sources of phylloquinone. Rapeseed and soybean oils have been reported to contain more than 100 μg phylloquinone per 100 g (Zonta and Stancher, 1985; Ferland and Sadowski, 1992b; Weihrauch and Chatra, 1993; Moussa et al., 1994; Gao and Ackman, 1995; Shearer et al., 1996; Cook et al., 1999). Phylloquinone levels are also moderate in olive oils (49-82 μg/100 g), whereas other oils, such as sunflower and maize oils, contain less than 10 μg/100 g (Ferland and Sadowski, 1992b; Weihrauch and Chatra., 1993; Jakob and Elmadfa, 1996; Shearer et al., 1996; Cook et al., 1999). A great variation seems to occur also in the phylloquinone contents of oils; probably as a consequence of differences in the industrial methods and raw materials used to produce them (Zonta and Stancher, 1985; Gao and Ackman, 1995). In the case of margarines the fat content and the raw materials in the products greatly affect the phylloquinone content, generally moderate values have been reported for various margarines consumed in the USA (15-161 μg/100 g) (Weihrauch and Chatra, 1993; Booth et al., 1995; Cook et al., 1999).

As already mentioned hydrogenated oils also contain 2',3'-dihydrovitamin K_1, a proportional amount of which increases with higher levels of hydrogenation, whereas the amount of phylloquinone decreases (Davidson et al., 1996). In a study by Booth et al. (1996b) dihydrovitamin K_1 was quantified in 36 prepared foods with a high fat content among a total of 261 foods analysed. They found the highest contents from various bakery products (21-59 μg/100 g) and in stick margarine (57 μg/100 g), whereas Cook et al. (1999) determined even higher dihydrovitamin K_1 amounts from different margarines (up to 235 μg/ 100g). The processing of root crops and animal products with vegetable oils increases their vitamin K content significantly mainly in form of dihydrovitamin K_1. For example the summarised amount of phylloquinone and dihydrovitamin K_1 in fast-food French fries was 40 μg/100 g (Booth et al.,

1996b) when cooked potatoes contained only 1 μg/100 g (Booth et al., 1995). On the other hand, it has been suggested that dihydrovitamin K_1 has less biological activity than phylloquinone (Booth et al., 2000b).

Langenberg et al. (1986) investigated the effects of different processing techniques on the phylloquinone contents in vegetables. They observed that cooking or γ-irradiation hardly destroyed phylloquinone at all. The phylloquinone contents of deep-frozen or canned products were also the same as those in fresh vegetables. In agreement with this, a study by Ferland and Sadowski (1992b) indicated that phylloquinone in oils is also quite stable to heating; decrease in phylloquinone content was 7% in 20 min and 11% in 40 min at 185-190°C. On the other hand, light destroys phylloquinone very easily; exposing rapeseed and safflower oils to fluorescence or daylight for two days led to 46-59% and 87-94% destruction of the compound, respectively (Ferland and Sadowski, 1992b). Both Ferland and Sadowski (1992b) and Moussa et al. (1994) have, however, observed that this decomposition can be prevented by using amber bottles or by storing oils in the dark.

2.4.2 Foods of animal origin

In general, only scattered information about the K vitamers found in foods of animal origin is available; the current data is mostly limited to phylloquinone. Milk and infant formulas have been investigated more frequently than other items in this group, mainly because newborn infants have the greatest risk of serious vitamin K deficiency. The reported phylloquinone contents for cow milk are low (0.14-3.7 μg/100 mL) (Isshiki et al., 1988; Shino, 1988; Hirauchi et al., 1989b; Weihrauch and Chatra, 1993; Booth et al., 1994, 1995; Indyk and Woollard, 1995, 1997; Shearer et al., 1996). Both the season and the breed of cattle have been shown to affect the phylloquinone content of milk (Haroon et al., 1982; Fournier et al., 1987; Indyk and Woollard, 1995). In a few studies (Isshiki et al., 1988; Shino, 1988; Hirauchi et al., 1989b; Indyk and Woollard, 1997) also menaquinones were included in the analysis and MK-4 was found generally in amounts comparable to phylloquinone (0.4–1.2 μg/100 mL).

The vitamin K content of human milk is even lower; 0.03-1.3 μg/100 mL in the form of phylloquinone and 0.1-0.2 μg/100 mL as MK-4 (Haroon et al., 1982; Fournier et al., 1987; Isshiki et al., 1988; Hirauchi et al., 1989b; Canfield et al., 1990, 1991; Lambert et al., 1992; Pietschnig et al., 1993; Indyk and Woollard, 1995). Variations both between individuals and

between various stages of lactation have been reported (Haroon et al., 1982; Fournier et al., 1987; Canfield et al., 1990, 1991; Lambert et al., 1992; Indyk and Woollard, 1995), whereas maternal dietary intake of vitamin K seemed not to have any effect on the concentration (Pietschnig et al., 1993). The infant formulas are generally supplemented with phylloquinone and therefore contain significantly more vitamin K than human milk. The raw materials used, however, greatly influence their vitamin K contents; for example Indyk and Woollard (1997) found appreciable dihydrovitamin K_1 amounts in infant formulas containing hydrogenated oils. Generally only phylloquinone has been analysed in infant formulas; because of variations in the methods used to report the results, the exact comparisons of the results are difficult. Phylloquinone contents are typically 0.3-24 μg/100 mL for liquid samples and 21-141 μg/100 g for powders (Haroon et al., 1982; Bueno and Villalobos, 1983; Hwang, 1985; Landen et al., 1989; Tanner et al., 1993; Indyk et al., 1995; Zamarreno et al., 1995; Indyk and Woollard, 1997).

Among the other dairy products yoghurt contains phylloquinone at less than 1 μg per 100 g whereas the phylloquinone amounts in butter and various cheeses are generally 5-10 μg/100 g (Shino, 1988; Hirauchi 1989b; Weihrauch and Chatra, 1993; Booth et al, 1995; Shearer et al., 1996; Cook et al., 1999; Schurgers et al., 1999). The contents of menaquinones differs between various cheese types; values up to 80 μg/100 g have been published for dominating form, MK-8 (Hirauchi et al., 1989b; Shearer et al., 1996; Schurgers et al., 1999). In addition, high MK-4 and MK-7 contents (19.4 and 84.5 μg/100 g, respectively) have been reported for butter (Shino, 1988).

The phylloquinone contents of various meats are extremely low (< 1 μg/100 g) (Hirauchi et al., 1989a,b; Weihrauch and Chatra, 1993; Booth et al., 1995; Shearer et al., 1996; Schurgers et al., 1999). In a few studies, in which their menaquinone contents have been analysed, only MK-4 has been found in higher amounts, especially from chicken (1-30 μg/100 g) (Hirauchi et al., 1989a,b; Schurgers et al., 1999). Similarly, the contents of different K vitamers in fish were very low (< 2 μg/100 g) although some differences between species have been reported (Hirauchi et al., 1989b; Udagawa, 1993; Weihrauch and Chatra, 1993; Shearer et al., 1996; Schurgers et al., 1999). Both the phylloquinone and MK-4 contents are higher in the egg yolk (2-4 and 27-30 μg/100 g, respectively) than in the albumen (0.01 and 0.8-1 μg/100 g, respec-

tively) (Hirauchi et al., 1989b; Weihrauch and Chatra, 1993; Shearer et al., 1996; Schurgers et al., 1999).

3 OBJECTIVES OF THE PRESENT STUDY

The main objectives in the present study were to:

1) Develop methods for determining the phylloquinone and menaquinone contents in food materials.

2) Apply the developed methods for analysing the phylloquinone and menaquinone contents in various food groups.

3) Estimate the average dietary intake of vitamin K in Finland on the basis of the data produced.

4 MATERIALS AND METHODS

4.1 Sampling

The aims of the sampling were based on the ranking of food items in three categories. Both consumption statistics (Statistics Finland, 1993; Information Centre of Finnish Margarine Industry, 1996; Ministry of Agriculture and Forestry, 1996) and what is known about the vitamin K contents of foods were considered when selecting and ranking samples. The role of the food items in the first category as dietary source of vitamin K was regarded as minor and they were analysed only once as pooled samples. The items in the second category were also analysed as pooled but their sampling was repeated two or four times. The greatest effort was put into the sampling of food items, which were regarded as good sources of vitamin K and also consumed extensively in Finland. Their sampling was repeated at least once and in addition to pooled samples, also individual subsamples were analysed.

The food items were mostly purchased from 10 retail stores in the Helsinki area, representing the four major food chains in Finland, between winter 1996 and summer 1998. Samples were purchased in the units, in which they are usually sold, thus they represented the food obtained in the same manner by the consumers. The aim was to obtain 10 subsamples of each food item at one sampling time in order to prepare one composite sample. Samples were first subjected to normal household practises, e.g. peeling, so that only the edible part of food was

analysed. In addition, large samples were cut into, for example, quarters and diagonally opposite portions were taken. The samples were homogenised as little as possible before equal amounts (usually 100-150 g) of each subsample were added to the pool. The composited sample was mixed manually, vacuum-packed into plastic bags in small portions and stored in the dark at -20°C until analysis. Usually each analytical sample was weighed in its own separate bag to ensure that pooling was done properly.

Oils, margarines and butter (I, IV)

Samples of 14 different margarines varying in fat contents and in raw materials, butter and six different oils were purchased twice during the winter 1995-1996 from retail stores (I). The margarines chosen for study represented the most popular brands from both Finnish manufactures and the butter was from the main producer. The rapeseed, soybean and sunflower oils were produced by Finnish manufactures whereas olive oils were from Italy. The number of subsamples at one sampling time varied usually between 8 and 10, and the subsamples were pooled according to oil type or margarine brand. At the first sampling time six individual subsamples of rapeseed oil as well as of the most popular margarine brand were analysed separately. In addition, two batches of crude rapeseed oil and the corresponding refined oils from one Finnish manufacturer as well as two batches of crude rapeseed oils and the corresponding margarine compositions and margarines from another were analysed. All these samples were analysed only for phylloquinone.

In study IV both the phylloquinone and 2´,3´-dihydrovitamin K_1 contents of 12 samples were analysed during the winter of 1997. These samples included crude and hydrogenated rapeseed and soybean oils, hydrogenated rapeseed-palm oil mixture, four different household margarines and three different margarines produced for the baking industry. All the samples except for the three household margarines, which were purchased from 10 retail stores and pooled as described above, were obtained from two Finnish manufacturers. In the case of hydrogenated soybean oil and the mixture of rapeseed and palm oils two batches were analysed.

Vegetables, fruits and berries (II)

A total of 39 vegetables, fruits and berries were sampled between summer 1996 and the winter of 1997 prior to analysing their phylloquinone contents. In addition, two separate pools of broccoli, carrot, Chinese cabbage, cucumber, Iceberg lettuce and tomato as well as four separate pools of pot-grown lettuce and white cabbage were purchased at different times of the

year. Six individual subsamples of carrot, pot-grown lettuce and white cabbage were also analysed. The analysed samples, except fruits, were mainly domestic. Most of the samples were purchased from retail stores, but a number of berries and domestic apple samples were supplemented with items bought from market places and stands outside stores. Generally eight to ten subsamples weighing 0.2-1.0 kg were obtained of each food item.

Cereals (III)

Samples of selected milling and bakery products (14 in total) were obtained from retail stores during the winter of 1997. The subsamples purchased represented different Finnish manufacturers. The number of subsamples (0.2-1.0 kg) per one food item was from eight to ten, and only their phylloquinone content was determined.

Foods of animal origin (V)

The selected examples of meat, fish and dairy products (13 in total) were also purchased from 10 retail stores representing the main food chains during the summer of 1998. There were eight to ten subsamples weighing 0.2-1.0 kg of each food item in this group; all samples were domestic. Fish and meat samples were homogenised before pooling. Both the phylloquinone and menaquinone contents of these samples were analysed.

4.2 Vitamin K analysis

4.2.1 General principles of the methods

The vitamin K compounds of the pooled food items were isolated by using specific solvent extraction method for each food group, purified and analysed by HPLC. At least two extraction methods were tested for every food group; the experiments are summarised in Table 2. Semipreparative HPLC was used for purification of most of sample extracts. Vitamin K contents of plant foods were quantified with electrochemical detection (EC) whereas fluorescence detection (FL) after reduction with metallic zinc was used for analysis of the animal products. The analyses were mostly carried out in triplicate and quantification was based on the peak areas. All work was done under subdued light conditions.

Table 2. The summary of extraction and purification methods tested for different food groups

FOOD GROUP/ Test materials	**METHOD A**	**METHOD B**	**METHOD C**	**The selected method:** extraction + purification + quantification
Margarines/ 80% margarine 40% margarine	Extraction with hexane[a]	Extraction with 2-propanol-hexane[b]	Extraction with diethyl and petroleum ether after ammonia treatment[b]	Method A + semipreparative HPLC + HPLC with EC detector
Vegetables/ White cabbage Carrot	Extraction with chloroform-methanol[b]	Extraction with 2-propanol-hexane after digestion in 2-propanol[a,c]	B without digestion[b]	Method B ± semipreparative HPLC + HPLC with EC detector
			B after cooking in boiling water[b]	
Cereal products/ Rye meal Rye bread	Extraction with chloroform-methanol[b]	Extraction with 2-propanol-hexane after digestion in 2-propanol[a,c]		Method B + semipreparative HPLC + HPLC with EC detector
Meat products/ Bovine liver	Extraction with chloroform-methanol[b]	Extraction with 2-propanol-hexane after digestion in 2-propanol[a]		Method B ± lipase treatment + semipreparative HPLC + HPLC with FL detector
Dairy products/ Emmental type cheese	Acid hydrolysis method + lipase treatment[a,c]	Extraction with 2-propanol-hexane after digestion in 2-propanol[b] + lipase treatment[a]	Method A + liquid reductive extraction[b]	Method A ± lipase treatment + semipreparative HPLC + HPLC with FL detector
			Method B + liquid reductive extraction[b]	

[a] The exact procedure in text. [b] The exact procedures published in original papers (I-III,V).
[c] Different digestion times tested: see original papers (II, III and V).

The vitamin K contents were mainly quantified using the internal standard method; MK-4 (plant products) and $K_{1(25)}$ (animal products) were used as the internal standards. To confirm the validity of use of the internal standards a blank sample, in which no internal standard was added, was done first for all samples. Blank tests of butter, blended margarines, Danish pastry, Karelian pie, doughnuts and sweet wheat bread showed that they naturally contained MK-4, which was used as the internal standard. Therefore, the phylloquinone contents of butter and these bakery products were quantified by the external standard method with recovery correction, whereas the phylloquinone contents of blended margarines were quantified by the internal standard method taking into account their small portions of endogenous MK-4. The external standard method was also used in study IV for quantification of phylloquinone in margarines, which contained no dihydrovitamin K_1.

4.2.2 Standards (I-V)

The phylloquinone and MK-4 standards were obtained from Sigma Chemical Co., USA. The 2´-3´-dihydrovitamin K_1, $K_{1(25)}$ and MK-5 to MK-10 standards were received as gifts either from Hoffman-La Roche and Co., Switzerland or from Eisai Co., Japan. The stock and working standard solutions were prepared such that convenient volumes (0.5-1 mL) could be used when adding the vitamins to the samples as internal standards or for the recovery tests. The *n*-hexane solutions of standards were stored at -20°C in the dark for up to four week, whereas the working standard in the mobile phase was prepared weekly. However, in the menaquinone study (V) the preservation times were longer (up to 2 months). The concentrations of phylloquinone, MK-4 and $K_{1(25)}$ solutions were confirmed by analysing them spectrochemically at 249 nm ($E^{1\%}_{1cm}$=419, Merck).

4.2.3 Sample preparation

Oils, margarines and butter (I, IV)

In the case of fat samples phylloquinone and dihydrovitamin K_1 were extracted with *n*-hexane. The weighed oil samples (0.5-1.0 g) were diluted together with the internal standard (MK-4) to 10 mL of *n*-hexane, whereas margarines and butter were shaked for 1 min in approximately 5 mL *n*-hexane before dilution to a volume (10 mL). Margarine extracts were allowed to stand for 30 min before a 2-mL aliquot was evaporated under nitrogen and the residue dissolved in *n*-hexane. Semipreparative HPLC was used for purification of both sample types.

Vegetables, fruits, berries and cereal products (II, III)

The phylloquinone was extracted from plant food items with 2-propanol/hexane (a modification of the method of Langenberg et al., 1986). In this method the homogenised sample (2-3 g) was weighed into a centrifuge tube, after which the internal standard (MK-4) and 10-15 mL of 2-propanol were added. The samples were digested in a boiling water bath (root crops and cereal products 10 min, other items 5 min). After cooling, 10 mL of 2-propanol was added and the sample was homogenised with an Ultra-Thurrax T25 mixer for 2 min. Homogenisation was repeated twice after adding 10 mL of *n*-hexane. Finally, 10 mL of water was added and after rapid shaking the extracts were centrifuged at 1500 g for 5 min. An appropriate amount of *n*-hexane phase was evaporated to dryness under nitrogen, redissolved in *n*-hexane and purified by semipreparative HPLC. In the case of green vegetables the residue was, however, dissolved straight in the mobile phase of analytical HPLC because purification was unnecessary.

Meat and fish products (V)

For the extraction of phylloquinone and menaquinones from meat and fish products the 2-propanol/hexane extraction method described above was used. Otherwise the procedure was the same (digestion time 5 min), except that $K_{1(25)}$ was used as the internal standard. Similarly with plant food items also these extracts were purified by semipreparative HPLC, after lipase treatment in the case of rainbow trout.

Dairy products (V)

Acid hydrolysis method (a modification of the AOAC method, 1990) was used to extract phylloquinone and menaquinones from dairy products. The sample (3 g) was weighed into a Mojonnier flask, and the internal standard ($K_{1(25)}$) as well as 10 mL of H_2O and 10 mL of HCl (37%) were added. The contents of flasks were mixed and immersed in a boiling water bath for 10 min. After cooling the extraction was done with diethyl ether and petroleum ether (1:1) three times; firstly with 25 + 25 mL and then twice with 15 + 15 mL (shaking for 1 min each time). The combined extracts were evaporated using a rotavapor and purified with lipase treatment and further by semipreparative HPLC; soured whole milk and yoghurt were only purified chromatographically.

4.2.4 Purification

Semipreparative HPLC (I-V)

The straight-phase semipreparative HPLC was used for purification of almost all the sample extracts after filtering the extracts through a membrane filter (Puradisc 25 TF 0,45 μm; Whatman, USA). The HPLC apparatus consisted of a Waters Model 510 HPLC pump (Waters Associates, USA), a Rheodyne 7125 injector (Rheodyne, USA), a Merck-Hitachi L-4200 UV-VIS detector (Hitachi, Japan) set at 248 nm and an LKB 2220 recording integrator (LKB, Sweden). A μPorasil column (10 μm, 30 cm x 3.9 mm, Millipore, USA) after a Waters Guard-Pak holder with a silica insert (Millipore) was used to separate K vitamers. The mobile phase was 1% diethyl ether in *n*-hexane and flow rate was 1.5 mL/min. The retention times for various K vitamers were established several times daily with standards, and a collection time was from 1.5-2 min before first eluting compound (*cis* isomer of phylloquinone, except for dihydrovitamin K_1 in study IV) to 1.5 min after the elution of the last vitamin K compound (MK-4). After collection the vitamin K fraction was evaporated to dryness under nitrogen and dissolved in the mobile phase used in the analytical step.

Lipase treatment (V)

Enzymatic removal of fat (a modification of the method of Indyk and Woollard, 1997) was used as the first purification method for the rainbow trout and cheese samples. Lipase powder (2 g; Type VII from Candida cylindracea, L1754, Sigma) and 40 mL of phosphate buffer (0.8 M, pH 8) were added to dry extraction residues. The tubes were shacked, sonicated for 5 min and incubated at 37 °C for 120 min in a shaking water bath. After cooling, ethanol (20 mL) was added and the contents of the tubes were mixed. K vitamers were re-extracted twice for 2 min with 10 mL of *n*-hexane. Finally, the *n*-hexane phase was transferred to a flask and evaporated using a rotavapor. The residue was dissolved in *n*-hexane and purified further by semipreparative HPLC as described above.

4.2.5 Analytical HPLC

Quantification of phylloquinone and dihydrovitamin K_1 with EC detector (I-IV)

Quantification of phylloquinone and dihydrovitamin K_1 in plant food items was performed with reverse-phase HPLC with a dual-electrode EC detector. The method was based on that of Hart et al. (1985). The apparatus consisted of a Merck-Hitachi L-2000 pump (Hitachi), a Merck T-6300 column thermostat (Merck, Germany), a Waters 717 autosampler, an ESA

Coulochem II EC detector equipped with a guard cell (model 5020) and a dual-electrode analytical cell (5011) containing two porous graphite electrodes in series (ESA, USA) and Millenium 2010 chromatography manager (Waters). K vitamers were separeted with a Vydac TP54 column (5 μm, 250 x 4.6 mm) after they had gone through a C18 guard column (Waters, Nova-Pak). The column temperature was kept at 30 °C. The mobile phase consists of methanol (95%) and NaAc buffer (0.05 M, pH 3); the flow rate was 1.0 mL/min. The detector was operated in the redox mode, in which the upstream electrode (-1.1 V) reduced the vitamin K compounds and the downstream electrode (0 V) reoxidised them. The injection volume was 30 μL and the running time was 60 min. The vitamin K contents of the samples were quantified by the internal standard method (MK-4 as internal standard) based on the peak areas, in which the response factor was determined daily at one concentration level and monthly at three levels.

Quantification of phylloquinone and menaquinones with FL detector (V)

The phylloquinone and menaquinone contents of animal products were quantified with reverse-phase HPLC with a FL detector after post-column reduction with metallic zinc (a modification of the method of Haroon et al., 1987). The chromatographic apparatus, except for the detector, was the same as in the first quantification method; fluorescence was monitored using a Waters 470 fluorescence detector (Waters Associates, Milford, MA, USA) with excitation and emission wavelengths set at 238 nm and 425 nm, respectively. Fluorescent derivatives of K vitamers were produced online using a post-column chemical reactor (2.1 x 50 mm) packed with metallic zinc (particle size < 45 μm, Merck). The reactor was placed between the chromatography column and the detector. In addition, an oxygen-scrubber consisting of a stainless-steel column (4 x 125 mm) packed with 5% platinum-on-alumina (Merck) was connected between the pump and injector to increase the sensitivity of the method (Davidson and Sadowski, 1997). The mobile phase consists of 83% methanol and 17% ethanol containing 10 mM $ZnCl_2$, 5 mM NaAc and 5 mM acetic acid. Separation of phylloquinone and menaquinones was achieved with a Vydac TP54 column by isocratic elution starting with a flow rate 0.8 mL/min. After 8 min the flow rate was increased to 1.5 mL/min in four minutes; the 1.5-mL/min flow rate was maintained for 28 min before returning back to the initial conditions. The injection volume was 50 μL. The vitamin K contents of the samples were quantified by the internal standard method ($K_{1(25)}$ as internal standard) based on the peak areas, in which the response factor was determined daily at three concentration levels.

4.3 Method validation

4.3.1 Optimisation procedures (I-III, V)

Extraction conditions (Table 2) were optimised for various food groups to achieve maximum vitamin K extractability and stability. In addition, various methods to purify animal food extracts were tested, whereas the semipreparative HPLC was the only method tested to purify plant food extracts. When estimating the efficiency of the extraction and purification methods vitamin K contents, recovery of the internal standard and repeatability of the results were monitored. After selecting the Vydac TP54 column in preliminary studies, the separation of phylloquinone and MK-4 in plant food items as well as the separation of menaquinones from interfering compounds in animal products were optimised here. Moreover, optimal detection conditions, including the selection of applied potentials in EC detector and the wavelengths in FL detector were looked for during this study.

4.3.2 Method reliability (I-V)

The accuracy of the selected methods was tested in the same way by recovery tests for every food group. The recovery of phylloquinone calculated by the internal standard method was tested for various plant food items with used extraction methods. The recoveries of phylloquinone, MK-4, MK-8 and MK-9 calculated by the internal standard method for animal food items were monitored in a similar way. In both cases the recoveries of these analytes were also calculated by the external standard method and compared with that of the internal standard used in each method (MK-4 or $K_{1(25)}$). The recovery of the added internal standard was also monitored in all samples analysed. The repeatability of the methods was studied by following the coefficient variation (CV) of replicated analysis of the reference samples in studies I-III. The reference samples were randomly chosen food items of the food group under study, and their phylloquinone content were analysed in duplicate in every second series. Because each analytical sample was weighed in its own separate plastic bag, the CV for triplicated analysis describes both the repeatability of the method and pooling system.

The linearity ranges of the standard curves of analysed vitamin K forms as well as their detection limits were determined for both analytical HPLC systems. In addition, daily variations in detector responses and retention times were monitored with standard injections

after every third sample. Standard injections were also used in semipreparative HPLC to confirm the collection times of vitamin K fraction.

Identification of K vitamers was usually based on their retention times in the analytical HPLC. In addition, some techniques were used to confirm identification; with the EC detector the peak purity was established by comparing the response ratios of one oil and one margarine sample and the standard solution at two downstream electrode potentials (I). In the study V HPLC-MS was used to confirm the identification and quantification of most of the samples (bovine and pig livers, chicken meat, soured whole milk and all cheese samples). The separation of phylloquinone and menaquinones was based on the analytical HPLC procedure used with the FL detector. The mass spectrometric detection was carried out with an ion-trap mass spectrometer (Esquire-LC, Bruker Daltonik, Bremen, Germany) using positive ion atmospheric pressure chemical ionisation (APCI). Selected ion monitoring for protonated K vitamers was performed by measuring the intensity of mass - charge ratios of 445, 451, 513, 521, 581, 649, 717, 785 and 853 for $[MK4+H]^+$, $[K1+H]^+$, $[MK5+H]^+$, $[K1(25)+H]^+$, $[MK6+H]^+$, $[MK7+H]^+$, $[MK8+H]^+$, $[MK9+H]^+$ and $[MK10+H]^+$, respectively. The used MS parameters are presented in paper V. Quantification was done using the internal standard method.

The accuracy of the HPLC systems was tested by participating in two collaborative studies. The first was the European Quality Assurance Scheme for vitamin K; the programme was started in 1996 and is still going on. In this program phylloquinone standards (and plasma samples) are analysed four times per year. The second was the AOAC collaborative study in summer 1998, in which phylloquinone contents of infant formulas were determined by a given method using FL detector after reduction with metallic zinc.

4.4 Moisture, fat and *trans* fatty acids analysis

The moisture content of all samples was determined by drying at 100 $\pm$ 2 °C to a constant weight (AOAC 952.08, modified; AOAC, 1990). The AOAC method was also used to determine the fat contents of the meat and fish samples (AOAC 948.15; AOAC, 1990). In study IV *trans* fatty acid contents of oil and margarine samples were determined as described by Hyvönen et al. (1993).

4.5 Calculations of the intake of vitamin K in the average Finnish diet

Data on the average food consumption by the Finnish population was mainly derived from the Food Balance Sheet (Ministry of Agricultur and Forestry, 1999) and from Statistics Finland (2000). In the case of vegetables details of consumption were taken from the Balance sheet for vegetables (Tikkanen, 1993), as well as consumption figures of oils, margarines and butter were obtained from Information Centre of Finnish Margarine Industry (2000). When estimating vitamin K intakes, the average vitamin K contents (summarised amount of various forms for animal products) determined in this study were used, except for milk and eggs whose vitamin K contents were taken from studies of Booth et al. (1995) and Hirauchi et al. (1989b), respectively (Table 3). Generally estimation was done separately for each food item and the total intake of the food group was calculated as the sum of these values. However, in the case of milling and bakery products as well as various cabbages and lettuces the estimation was based on the average vitamin K content in each group. Moreover, the average value of various meats determined here was used when estimating vitamin K intake from meat products, such as sausages.

5 RESULTS

5.1 Method validation

5.1.1 Optimisation of the method

When testing alternative extraction methods for analysing vitamin K in various foods, significant differences in vitamin K contents or in recoveries of the internal standards were seldom observed. Thus, the main criteria in the selection of the method for routine analysis was usually the repeatability of the results and the simplicity of the method. In the case of vegetables digestion of samples in 2-propanol prior to extraction with the mixture of 2-propanol and *n*-hexane was observed to be useful; better extractability was not, however, achieved after cooking in boiling water. The other samples, except for oils and margarines, were also digested in 2-propanol or HCl (dairy products) before extraction. Although the optimal digestion time varied between groups, the effect of digestion time on vitamin K contents or the recovery of the internal standard was generally minor. Only in the case of

dairy products did prolonging the digestion in HCl seem to destroy vitamin K. The exact results of method comparisons are reported in original papers (I-III, V).

Green vegetables could be analysed directly after extraction; the purification of other plant food items was made successfully with semipreparative HPLC. Its efficiency was also enough for animal products with low fat content, whereas food items with high fat content (>10 %), cheese and rainbow trout, needed another purification step. After a comparison of the two methods lipase treatment was selected for the first purification method of these items.

The quantitative analysis of vitamin K was made with reverse-phase HPLC systems; the Vydac TP54 column was used for separation. In the case of plant food items good separation of phylloquinone and MK-4 was achieved with isocratic elution (95% MeOH/0.05 M NaAc as the mobile phase). Optimal conditions for quantification of plant products were selected by determining the voltammograms of the EC detector; the best signal-to-noise ratio was obtained by using potentials –1.1 V (upstream electrode) and 0 V (downstream electrode). When analysing animal food products the detection system was changed to the fluorescence detector, in which the best response was achieved by using wavelengths 238 nm and 425 nm. The use of methanol-ethanol (83:17) as mobile phase was observed to result in good separation of phylloquinone and menaquinones; a flow gradient was used to reduce the broadening of the later peaks.

5.1.2 Method reliability

When testing the accuracy of the methods for vitamin K analysis in different food groups, recoveries of (calculated with the internal standard method) 92-102% for phylloquinone and of 60-92% for MK-4, MK-8 and MK-9 were achieved. The overall recoveries (calculated with the external standard method) were 78-107% for phylloquinone, 71-100% for MK-4, 51-56% for MK-8 and MK-9 and 70-73% for $K_{1(25)}$ when tested with 14, 12, 2 and 2 different matrixes, respectively (I-III, V). In routine determinations the recovery of the internal standard was usually 75-100% (MK-4) or 65-90% ($K_{1(25)}$). The coefficients of variation (CV) for the triplicated samples were <10% for phylloquinone and <15% for various menaquinones. When analysing vitamin K contents near the detection limit or for very heterogeneous samples, the CV was higher (10-22%). The CV for the replicated analysis of reference samples was generally <7%.

The response of the EC detector was linear for phylloquinone and MK-4 over tested range: 0.1-50 ng per injection (the coefficient of correlation 0.9992). The detection limits (defined as a signal twice the height of the noise level) were 50 pg for phylloquinone and 20 pg for MK-4. Respectively, the detection limits of the fluorescence detector were from 25 pg (phylloquinone and MK-4) to 400 pg (MK-10) and the tested linear range for various forms was 0.1-15 ng per injection the coefficient of correlation being 0.9991. The daily variation in the detector response for various forms was mostly <4% in both detection systems. The day-to day variation was slightly higher; 6-10% with the EC detector and 9-18% with the FL detector.

In the study I two reoxidizing potentials were compared. The equal response ratios of phylloquinone and MK-4 in the standard solutions and in the samples indicated the peak purity. In study V the identification of K vitamers in animal food items was confirmed with HPLC-MS and generally good agreement between fluorescence and MS data was achieved. However, a few disagreements were also observed; MK-5 in bovine liver and MK-5, MK-8 and MK-9 in Emmental type cheeses were found by FL detector but were not detected with HPLC-MS. On the other hand, MS analysis confirmed the presence of MK-6 and MK-7 in Emmental type cheeses although their concentrations were below the quantification limit of the FL detector. The detected K vitamers were quantified from HPLC-MS at the same level as they were determined with the fluorescence detector.

In addition, the accuracy of the detection systems was confirmed in collaborative studies. When analysing phylloquinone standards (seven times) our result was generally in the acceptable range (mean $\pm$ 2 SD) compared with the results of the laboratories (8-12) participating in the programme. The accuracy of our result was the same regardless of the detection system used (EC or FL) or the concentration level of the standard. In the AOAC collaborative study 2 milk and 6 infant formula samples were analysed; our results were mostly very similar to the average value of the 33 laboratories. Repeatability of our results for blind dublicates was comparable to repeatability of other participating laboratories (Indyk and Woollard, 2000).

5.2 The vitamin K contents in foods

Oils, margarines and butter (I, IV)

Among the oils analysed the highest phylloquinone contents were found in rapeseed and soybean oils (117-158.5 μg/100 g) whereas the contents were moderate in olive oils (25.1-50 μg/100 g) and low in sunflower oil (9.2-10 μg/100 g). On the other hand, relatively high phylloquinone amounts were found in soft margarines with 80% fat content (82-110 μg/100 g). Among the margarines phylloquinone content correlated with the plant fat content, therefore the phylloquinone contents were lower in soft margarines with lower fat content (45-104 μg/100 g) and in blended and hard margarines containing also animal fat (30-90 μg/100 g). The lowest phylloquinone amount was found in butter (6-8 ug/100g).

The variation between two sampling times was 0-27% for different oils and 0-36% for various margarine brands. Similarly, a variation was observed when individual subsamples were analysed. The phylloquinone contents of six rapeseed oil bottles and six margarine packages varied between 140 and 187 μg/100 g (CV 11%) and 96 and 117 μg/100 g (CV 6.6%), respectively. The fairly high variation was also observed in processing samples; 14% between four batches of the crude rapeseed oil and 12% between two batches of margarine. Whereas a 20 % loss was observed in the phylloquinone content of rapeseed oil after refining, no phylloquinone losses were found to occur during the margarine production. The phylloquinone content in the margarine sample obtained right after production (the process sample) was, however, 13% higher than in a retail sample of the same brand. The comparison of the refined and cold-pressed unrefined oils resulted in non-systematic results; the phylloquinone content was higher in the cold-pressed olive oil whereas the reverse was true in the case of rapeseed oil.

In addition to phylloquinone, dihydrovitamin K_1 contents were determined in a few oil and margarine samples. Among the oils dihydrovitamin K_1 was found only from hydrogenated oils (100-155 μg/100 g), in which it accounted for 47-72% of the sum of phylloquinone and dihydrovitamin K_1. Generally this sum was similar to the phylloquinone content in the corresponding crude oils. Variations in dihydrovitamin K_1 proportions between two batches of a mixture of rapeseed and palm oils as well as of soybean oil were under 10%. Dihydrovitamin K_1 was not detected in household margarines, whereas in the margarines meant for

baking industry its proportion was approximately 25% (6-28 μg/100 g). *Trans* fatty acids were detected only in the oils and margarines that contained dihydrovitamin K_1.

Vegetables, fruits and berries (II)

Phylloquinone is the only existing vitamin K form in vegetables, fruits and berries; it was found in a wide concentration range (up to 360 μg/100 g). The highest phylloquinone amounts (> 200 μg/100 g) were determined from dark-green vegetables (parsley, dill, spinach and Brussels sprouts). The leaf lettuce, broccoli and pot-grown lettuce were also good sources of phylloquinone; their contents were over 100 μg/100 g. The phylloquinone levels in other green vegetables varied between 15 and 80 μg/100 g, whereas the contents were considerable lower both in red and yellow vegetables as well as in root crops (usually <10 μg/100 g). Similarly the phylloquinone levels in fruits and berries were low (<11 μg/100 g), except for green fruits and black currant, which contained 19-34.3 μg phylloquinone per 100 g. Peeling was observed to decrease the phylloquinone contents of cucumber and apple by 60%.

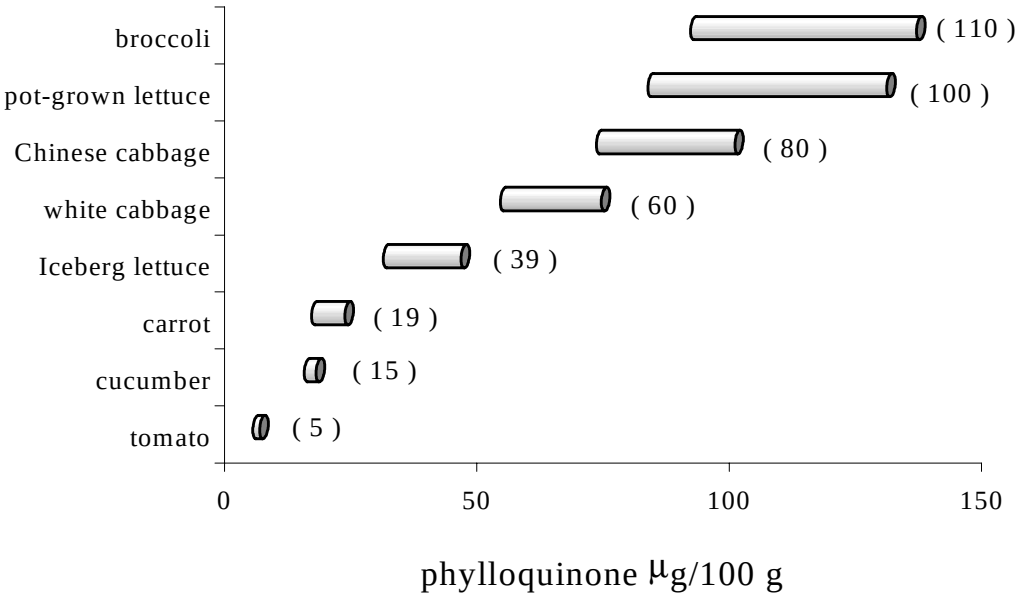

Figure 3. Range in the phylloquinone contents of the vegetables analysed two or four times. The average value of each item in parenthesis.

In the case of eight vegetables regarded as important sources of vitamin K sampling was repeated two or four times. Generally a moderate variation (2-18%) was observed between different sampling times although the variation was statistically significant only in the case

of pot-grown lettuce, Iceberg lettuce and carrot (Figure 3). Usually the higher amounts were found in samples representing the new crop of the year. The variations between individual samples of white cabbage, carrot and pot-grown lettuce were 42%, 14% and 31%, respectively.

Cereal products (III)

The phylloquinone levels were generally very low in all the cereal products analysed. Among the milling products the phylloquinone amounts ranged from 0.16 to 5.9 μg/100 g, whereas slightly higher amounts were found in bakery products (1.8-8.2 μg/100 g). The phylloquinone content seemed to correlate with the fat content of bakery products, thus the highest amount was found in raised doughnuts.

Foods of animal origin (V)

Both the phylloquinone and menaquinones (MK-4 to MK-10) were quantified from a few animal food items; the summarised vitamin K amounts of fish, meat and dairy products were generally very low (1-10 μg/100 g). Long-chain menaquinones (>MK-8) were found only in pig and bovine livers, Edam type cheese and soured whole milk in the range of 0.2-30 μg/100 g. Among the fish samples analysed the best vitamin K source was rainbow trout, which contained 3.1μg/100 g mainly in the form of MK-4. Similarly MK-4 was the dominant vitamin K form in different meats (beef, pork, chicken); the highest amount (60 μg/100 g) was found in chicken meat. Among the dairy products various cheeses contained vitamin K in the range of 7.8-49.4 μg/100 g; the distribution of menaquinones varied significantly between cheese types. The sum of different forms in soured whole milk was 8.4 μg/100 g, whereas yoghurt contained vitamin K only as phylloquinone and MK-4 (0.7 μg/100 g).

5.3 Dietary sources of vitamin K in the average Finnish diet

The total daily intake of different forms of vitamin K in Finland was estimated to be 120 μg, of which phylloquinone accounts for 80% of the total. The estimated intakes from various food groups are summarised in Table 3. Vegetables (43 μg per day) and oils and margarines (37 μg per day) were the most important food groups in vitamin K nutrition whereas the role of other plant food items, berries, fruits and cereals, was minor. The top five contributors of dietary vitamin K were soft margarines with 80% fat content, various

cabbages, rapeseed oil, lettuces and carrot. Animal products were the only source of dietary menaquinones in Finnish diet; the best sources were chicken meat (7.3 μg per day) and cheese (9 μg per day).

6 DISCUSSION

6.1 Representativeness of sampling

The whole sampling system including documentation of every step was carefully considered and performed according to principles presented by Greenfield and Southgate (1992) and procedures used in previous vitamin studies performed at our department (Piironen, 1986; Heinonen, 1990; Mattila, 1995; Vahteristo, 1998; Ollilainen, 1999). Food items were first ranked in the order of their priority in vitamin K nutrition. Mostly the composite samples were used to produce relatively large database. The sampling of food items regarded as the most important sources of vitamin K was repeated two or four times. Furthermore, some items within each group were selected for more detailed study and individual subsamples of them were analysed. Thus the suggestions of Greenfield and Southgate (1992) as well as those of Stewart (1995) were put into practice and information on variation provided in the given resources.

When using composite samples the general criteria deal with the number of primary samples, sample handling (including the size of subsamples used for compositing) and sampling plans (Greenfield and Southgate, 1992; Holden et al., 1997). In addition, both the collection of the samples and every step in sample handling should be documented carefully (Holden et al., 1997). The forming of composite samples was done according to these criteria. The main food chains were roughly sampled according to their market share and brand effects were included subjectively by sampling the most popular brand the most. Generally, the number of subsamples was ten, which is the recommended minimal number.

Table 3. The dietary intake of vitamin K from various food groups

Food group / item	Vitamin K content µg/g[a]	Consumption g/day	Intake of vitamin K µg/day[b]
Berries	**0.06-0.3**	**31**	**4.0**
Fruits	**0-0.34**	**119**	**4.8**
Vegetables			
Various cabbages	0.8-1.65	24	15
Various lettuces	0.4-1.6	5.5	5.5
Carrot	0.19	24	4.6
Tomato	0.05	28	1.4
Potato	0.01	180	1.8
Total		**322**	**43**
Cereals	**0.002-0.06**	**74**	**1.9**
Bakery products	**0.03-0.08**	**137**	**5.7**
Dairy products			
Soured whole milk	0.08	14	1.2
Yoghurt	0.007	45	0.3
Cheese	0.08-0.49	41	9
Total[c]		**561**	**11**
Eggs	**0.005[d]**	**23**	**0.1**
Fish	**0.01-0.04**	**34**	**0.7**
Meat products			
Chicken meat	0.6	12	7.3
Other meat	0.03-0.04	28	1.0
Liver	0.08-0.14	0.6	0.1
Total		**165**	**12**
Fats			
Butter	0.07	9.3	0.7
Margarines with 80% fat content	1.0	17	17
Other margarines	0.4-0.9	12	7.4
Rapeseed oil	1.5	7.1	11
Other oils	0.1-1.45	2.2	1.4
Total		**48**	**37**

[a] The analysed concentration range of the group / item
[b] The calculation of intakes: see 4.5 (page 48)
[c] The result for milk from the study of Booth et al. (1995)
[d] The result from the study of Hirauchi et al. (1989b)

The structure of the food markets and the efficient marketing systems in Finland make it possible to collect national representative samples of many food items from carefully selected retail stores in only the Helsinki area. For example oils, margarines and flours of the same few manufactures are sold all over the country. Similarly, imported vegetables and fruits are distributed through the same wholesalers to various parts of Finland. Thus sampling from different parts of the country was not regarded as necessary, although it has been recommended that samples should be taken from various geographical locations (Holden, 1997). In the case of meat, fish and domestic vegetables, fruits and berries the collecting area may affect vitamin K contents. Due to moderately low vitamin K contents in most of these items, it was not considered feasible to carry out a more comprehensive study at the beginning of vitamin K studies in Finland. However, at least in the case of green vegetables the effect of geographical location on their vitamin K contents is worth of further investigations.

The processing of samples was done immediately after purchase in reduced light to prevent the loss or degradation of vitamin K. After careful documentation of the product characteristics, equal amounts (100-150g) of each subsample was added to composite sample. In addition, special attention was focused to how this proportion was taken because of reported differences in vitamin K content for example in outer and centre leaves of white cabbage (Ferland and Sadowski, 1992a). Because of the heterogeneity of the certain samples each analytical sample was generally weighed from its own separate portion to be enable to evaluate the pooling and homogenisation practices.

Criteria set specially for sampling in vitamin K analysis (Booth et al., 1993) were taken into account as well as possible in the given resources. According to Booth et al. (1993) and Booth and Sadowski (1997) one of the problems in previous studies is inadequate sample description, thus the documentation of samples was done here carefully to facilitate the use of the analysed vitamin K values in food composition tables. Although our sampling system can be regarded as adequate at this stage of vitamin K studies in Finland, this study also showed the need for more detailed sampling.

6.2 Quality of the methods for determining vitamin K in foods

Although HPLC has been used in vitamin analysis since its introduction, naturally existing phylloquinone in foods has not been determined more often until in the 1990s. Still the number of studies published is minor, and even fewer deal with menaquinones. In previous studies a number of sample extraction methods have been used as summarised by Booth and Sadowski (1997). The documentation of validation and optimisation procedures of extraction, purification and detection steps is in many studies inadequate. The only existing standard method is the AOAC method (Indyk and Woollard, 2000; AOAC, 2000) for determining phylloquinone in milk and infant formulas; vitamin K content of these items has been actively researched.

In this study HPLC methods for determining phylloquinone and menaquinones in various food matrices were developed. Every step in sample preparation was modified and optimised separately for each food group. In addition, special attention was focused on the identification and quantification of K vitamers. Thus different detection systems as well as internal standards were used for plant and animal products: the electrochemical detector and MK-4 for the former and the fluorescence detector and $K_{1(25)}$ for the latter.

The general aim of the method development was to find a method, by which vitamin K can be extracted as efficiently as possible with minimal losses. The final extraction method was chosen separately for every food group after testing two or three extraction methods (Table 2). Although the differences between various extraction solvents tested were usually insignificant, in the case of plant foods the destroying of cell walls was noticed to be critical. This was successfully done with digestion in 2-propanol in a boiling water bath before extraction and by homogenisation (Ultra Thurrax) during extraction. In addition to results of this study, Langenberg et al. (1986) have proved the efficiency of the Ultra Thurrax mixer in extraction of vitamin K from the cells of vegetables. Digestion was also used for animal products to achieve an efficient extraction. The good recoveries of the internal standards confirmed that the digestion did not destroy vitamin K. In addition, the equal extractability of endogenous K vitamers and the internal standards was confirmed by similar ratios of analytes and internal standards in the first and the second extractions in the selected methods.

Due to low vitamin K contents and complexity of the sample items, careful purification of sample extracts was required. When reverse-phase chromatography was the method of choice for the final analysis, the selection of straight-phase chromatography for purification was reasonable. In addition to maximal separation capacity achieved by using two different chromatography types (Canfield et al., 1990; Lambert and de Leenher, 1992), it was easy to first use the straight-phase system, for example the dissolution of extracts containing fat to *n*-hexane was possible and the evaporation step after purification was rapid. This study showed the suitability and efficiency of semipreparative HPLC for purification; only in the case of animal food items with high fat content (>10%) was another purification step needed. After comparison of lipase treatment and liquid-phase reductive extraction, the former was selected due to the good recoveries and repeatability of the results achieved with this method. On the other hand, green vegetables could be analysed directly after removal of water-soluble compounds during the extraction. Other vegetables, however, required purification with semipreparative HPLC because coelution of an interfering compound with the internal standard in the analytical HPLC.

The straight-phase semipreparative HPLC separated *cis* and *trans* isomers of phylloquinone, whereas various menaquinones eluted close to each other allowing the use of reasonable short collection time (8 minutes in maximum). Another benefit of this method was the possibility to modify the purification according to samples, for example by changing the injection volume or collection time. On the other hand, this step was also critical due to variation in the retention times of the vitamin K compounds. Thus the collection times have to be verified regularly with standard injections. Because of the low sensitivity of the UV detector for K vitamers, large amounts of standards have to be injected and as a consequence of this a large volume of *n*-hexane has to be injected before the next sample to avoid a carry-through effect. In addition, the separation capacity of the column has to be maintained by washing it regularly although nonpolar impurities eluted first without accumulating to column.

The suitability of reverse-phase HPLC, when 95% methanol containing 0.05 M NaAc buffer was used as the mobile phase, for separation of phylloquinone and MK-4 in plant food items was proved in studies I-IV (see chromatograms in original papers). The starting point was to be able to use the same system also for animal products. However, it was not possible to practically separate menaquinones with considerable polarity scale with iso-

cratic elution. Thus, the EC detector has to be changed to a FL detector, where it was possible use the gradient elution or change flow rate during the chromatographic run. Good separation of phylloquinone and different menaquinones was achieved by using methanol-ethanol (83:17) as the mobile phase and by changing the flow rate during the elution (see chromatogram in paper V). The retention times were quite stable in both HPLC systems which indicates that the separation was repeatable.

The detection limits for various vitamin K forms (20-400 pg per injection) were similar with earlier studies (Hart et al., 1985; Haroon et al., 1987) and the sensitivities of both detectors (EC and FL) were sufficient for vitamin K analysis. According to small variations observed in the detector responses, both detectors seemed to function in a repeatable manner. In addition, the methods were selective enough, because quantification was based on the reduction of quinone forms to corresponding hydroquinones (Eitenmiller and Landen, 1999). Although the identification of the peaks based on the retention times was usually regarded as adequate, some techniques were used to confirm this. In the case of fat samples the equal response ratios of the samples and the standards at two potentials of the EC detector indicated the peak purity. The HPLC-MS data, however, showed that it is not possible unambiguously identify the menaquinones in animal products only using a fluorescence detector. The results from two collaborative studies confirmed furthermore the accuracy of the detection systems.

Good recoveries of the compounds obtained by the internal standard method were an indication of the accuracy of the selected methods. The reliability of the method was further confirmed by low day-to-day variation in the reference sample analysis and by the generally low variation in triplicate analysis. However, a greater variation was acceptable when analysing vitamin K contents near the detection limit or quantification was made with the external standard method. When replicated samples of heterogeneous food items were weighed from different plastic bags, the variation originates in both the analysis and the pooling system.

Due to the many steps in sample preparation, the use of internal standard was thought to be necessary; the need for the internal standard in vitamin K analysis was also suggested by Booth and Sadowski (1997). In addition to compensation for losses in the extraction and purification steps, the internal standard method takes into consideration the reduction efficiency of the detection systems. The selection of internal standard was, however, difficult

because only a few compounds with similar properties than vitamin K are available commercially. In addition, the distribution of various K vitamers differs between food groups (Booth and Sadowski, 1997). Therefore, in this study two internal standards: MK-4 for plant food items and $K_{1(25)}$ for animal food items, were used. Blank tests were used to determine if an internal standard could be used for every item. Moreover, the recoveries of analytes and internal standard calculated by the external standard method were close enough to prove their similar behaviour during the analysis. In addition, the reported recoveries of internal standards (65-100%) corresponded well to those previously reported (Booth et al., 1994).

The method developed suited well for determing vitamin K contents in various food groups. The repeatability of the methods was generally good although the uncertainty levels increased when working at levels which approached detection limits (as observed also by Booth et al., 1994). The selectivity of the EC detector for analysing phylloquinone was proved to be sufficient, whereas the reliable identification of menaquinones needed the use of HPLC-MS in addition to the FL detector. This study showed that vitamin K analysis is quite complicated due to many steps required during sample preparation. In addition, a universal method for all food items could not be found. The validation and documentation of the methods were done as carefully as possible; the used procedures fulfilled for the most part the criteria proposed for food composition studies (Booth et al., 1993; Holden et al., 1997). The existence of an official reference material for vitamin K would improve the quality control further.

6.3 The vitamin K contents in food items

Oils, margarines and butter (I, IV)

It was shown here that rapeseed and soybean oils are major sources of phylloquinone in Finland whereas the role of soybean and sunflower oils seems to be minor. The phylloquinone contents analysed here for various oils are compared to the previous results in Table 4. Although the reported concentrations, especially for rapeseed and soybean oils, lie within a wide range, there is some general agreement between the studies. The rapeseed oil analysed here was mainly Finnish turnip rapeseed oil (*Brassica rapa* subsp. *oleifera* DS), whereas in other studies rapeseed oil means usually canola oil. In agreement with this study, Gao and

Ackman (1995) noticed that refining decreases the phylloquinone content of rapeseed oil. When individual rapeseed oil bottles were analysed in present study, no correlation was found between storage time and the phylloquinone content. It had previously been reported that oils stored so that they were exposured to light have the reduced phylloquinone content (Ferland and Sadowski, 1992b; Gao and Ackman, 1995).

In this study, a significant variation was observed in the phylloquinone contents of individual rapeseed oil bottles as well as between two sampling times of crude oils and pooled oil samples. As stated both here and in other studies there are several possible reasons for this, for example differences in raw materials, season and storage conditions. Although non-consistent results were obtained here for the refined and cold-pressed unrefined oils, probably also the processing technique may have an impact. On the other hand, no systematic difference was observed between rapeseed oil of two Finnish manufacturers although both Zonta and Stancher (1985) and Ferland and Sadowski (1992b) have reported different phylloquinone values for various brands of oils.

Table 4. The phylloquinone contens (μg/100 g) in various oils

	1	2	3	4	5	6	This study
Rapeseed oil		114-188		81-348	123	98	117-160
Soybean oil	121-333	139-290	108-308	250	173	103	132-158
Olive oil		37-82			80	1.7	34-50
Sunflower oil		9					9.2-10

1) Zonta and Stancher, 1985 2) Ferland and Sadowski, 1992b 3) Moussa et al., 1994 4) Gao and Ackman, 1995 5) Shearer et al., 1996 6) Cook et al., 1999

Generally, moderate phylloquinone contents were found in various margarines; the content was high in soft margarines with 80% fat content. Generally our results were in good agreement with the few values previously reported although exact comparison was difficult because the documentation of the samples was not detailed enough in other studies and differences in margarine types between countries (Weihrauch and Chatra, 1993; Booth et al., 1995; Cook et al., 1999). Remarkable lot-to-lot variation and differences between various brands of the same type of margarine were observed both here and in the study of Cook et al. (1999). It seems that factors such as the oil composition of margarine, the degree of the

hydrogenation in oils, the quality of oil and storage conditions affect vitamin K content of margarine. As we also observed a moderate variation between individual samples of the same margarine brand, sampling and documentation of samples have to be done very carefully if a reliable database for margarines is to be produced.

According to our study only margarines meant for baking industry contained dihydrovitamin K_1 in Finland whereas in the USA significant dihydrovitamin K_1 contents in various margarines have been found (Booth et al., 1996b; Cook et al., 1999). The main reason for this difference is the effort of the Finnish margarine industry to reduce the amount of *trans* fatty acids in soft margarines. Thus, hydrogenated oils are not used in Finnish household margarines. Because *trans* fatty acid contents of margarines analysed elsewhere were not known, exact comparison of the results was difficult. However, we found dihydrovitamin K_1 from hydrogenated oils in the same proportions as Davidson et al. (1996).

The low phylloquinone content analysed here for butter is comparable to earlier results (Hirauchi, et al., 1989a; Weihrauch and Chatra, 1993; Booth et al., 1995; Shearer et al., 1996; Cook et al., 1999). In addition to phylloquinone, small amounts of various menaquinones have been quantified in other studies (Shino, 1988; Hirauchi et al., 1989b).

Vegetables, oils and berries (II)

Dark green vegetables were observed to be good sources of phylloquinone whereas it was found in moderate or low values in other vegetables. The results published here are generally within the ranges previously reported as illustrated for the items in Table 5. For most items the result of this study was near the minimal value of other studies although maximal value was approached in few cases, such as leek. For several vegetables analysed in the present study, e.g. Chinese cabbage and dill, no previous data were available. In addition, exact comparison of certain items, such as lettuces, is difficult due to the different varieties used in different countries.

The great variation observed here between different batches of the same vegetables as well as between individual samples is in agreement with findings of earlier studies (Ferland and Sadowski, 1992a; Booth et al., 1994). The highest phylloquinone contents were usually quantified in samples representing the new crop of the year; in the case of pot-grown lettuce, which is grown throughout the year in Finland, the highest phylloquinone level was found in May. In the case of heterogeneous items, such as leaf lettuce and white cabbage,

weighing of each analytical sample from individual plastic bag resulted in greater variation (> 10%) between parallel samples. This confirms the findings of Ferland and Sadowski (1992b); who observed that the outer leaves of cabbage contain 3-6 times more phylloquinone than the inner leaves. In addition, they found that the growth location and climate have a great effect on the phylloquinone content of vegetables. Other possible reasons for variations between individual samples as well as between results from different countries are season, harvesting time, storage and genetic factors. Thus the sampling should be regarded as a very important step when analysing phylloquinone contents of vegetables.

Table 5. The phylloquinone contens (μg/100 g) in the certain vegetables

	1	2	3	4	5	This study
Broccoli	205	178	147-230	113	179	91-136
Carrot	11		4-11		6	16-23
Cauliflower	25		5	20	31	20
Leaf lettuce	123	519-1180	210		129	160
Pea	33		33-39		34	28
Spinach	385	1001-1439	240-1220	360	380	270
Tomato	6		6-7	3	6	4.4-5.7
White cabbage	55	72-228	46-584		618	54-73

1) Langenberg et al., 1986 2) Ferland and Sadowski, 1992a 3) Weihrauch and Chatra, 1993
4) Booth et al., 1995 5) Shearer et al., 1996

Low phylloquinone contents were analysed here for various fruits and berries, except for green fruits and black currant, in which higher contents were found. Generally the results of this study are in agreement with previous studies (Weihrauch and Chatra, 1993; Booth et al., 1995; Shearer et al., 1996); the higher amounts were, however, found for grapes, kiwi fruit, blueberry and strawberry.

Cereal products (III)

The phylloquinone contents of cereals were low and their role in vitamin K nutrition was estimated to be minor despite the fact that they are consumed in large amounts. Although only phylloquinone contents were analysed here, some bakery products may also contain dihydrovitamin K_1 or menaquinones due to the fat used in their preparation. Estimates for

vitamin K in cereals obtained prior to this study are very limited; the reported phylloquinone contents are generally in good accordance with this study (Weihrauch and Chatra, 1993; Booth et al., 1995; Shearer et al., 1996). In addition, Booth et al. (1996) have found low dihydrovitamin K_1 amounts in bakery products containing fat. Probably mostly the same factors as in the case of vegetables influence variation in vitamin K contents of cereals although also milling practises may have some impact.

Animal products (V)

The vitamin K contents in animal foods were analysed with the methods, which were documentated and validated more carefully than in the previous studies. In addition to the method development, the aim here was to establish the presence of different menaquinones in various animal groups and get information for future studies. The lowest vitamin K amounts were found in fish, which did not contain long-chain menaquinones (>MK-8) at all. Among the meat products long-chain menaquinones were found only in liver, whereas MK-4 was the dominant form in various meats. As illustrated in Figure 4A, differences both in contents and distribution of various vitamin K forms were observed between animal species.

Figure 4. The distribution of various K vitamers in meat products (A) and in various cheeses (B).

Among the dairy products the vitamin K amounts were moderate in cheeses and soured whole milk whereas yoghurt hardly contained vitamin K at all. The long-chain menaquinones were, however, found only from soured whole milk and Edam type cheese. In the case of cheeses the cheese type have a greater effect on the distribution of menaquinones than the ageing time. The distribution of K vitamers in three cheeses is compared in Figure 4B.

When the results of this study are compared to previous phylloquinone values for animal products, it can be seen that a general agreement exists (Weihrauch and Chatra, 1993; Booth 1994, 1995; Jakob and Elmadfa; 1996; Shearer et al., 1996). In addition, the distribution of menaquinones is similar to other studies (Hirauchi et al., 1989a,b; Schurgers et al., 1999). Because the documentation of the sample characteristics is not sufficient in most of other studies, the comparison of vitamin K values was difficult. As this study indicated that there are differences especially between fish species and cheese types both in the contents and distribution of K vitamers, the careful documentation of sampling is important.

6.4 Vitamin K in the average Finnish diet

Based on the average per capita food consumption (Ministry of Agricultur and Forestry, 1999; Statistics Finland, 2000) and the results of this study, the average vitamin K intake from different foods was estimated to be 120 μg per day. This is 50% more than the current RDA recommendations (Food and Nutrition Board, 1989), which, however, may not be sufficient for both new and traditional roles of vitamin K (Vermeer et al., 1998). Moreover, a fairly high variation especially in the consumption of vegetables, which are regarded as the best sources of vitamin K, among individuals is to be expected. Thus the daily dietary intake of vitamin K may vary considerable. For example Booth et al. (1996a, 1999b) observed significant differences in dietary phylloquinone intakes between various age groups in the USA where the vitamin K intake is in general within the recommended daily intakes. On the other hand, Schurgers et al. (1999) reported daily average intake in the Netherlands to be as high as 250 μg whereas average intake in United Kingdom is reported to be only 64-72 μg/day (Bolton-Smith and Shearer, 1997).

Although the consumption of oils and margarines is fairly low in Finland, they are significant sources of vitamin K. The main reason for this is that the rapeseed oil, the phylloqui-

none content of which is high, is the most frequently used oil both in households and in the margarine industry. The contribution of oils and margarines is on average 30% of the daily intake. Even much higher intakes are, however, possible depending on individual dietary habits, for example, daily consumption of 60-70 g of soft margarine with 80% fat content would alone fulfil the recommendation.

Vegetables are another group, which is regarded as significant source of phylloquinone. Their role is enhancing further due to the increasing trend of their consumption during recent years. However, differences between individuals are high for the consumption of vegetables, thus vitamin K intake may vary highly. The recommended daily intake is easily satisfied for example by eating 50 g of lettuce, one carrot (70 g) and 50 g of black currant per day.

According to the results of this study dihydrovitamin K_1 may not play significant role in vitamin K nutrition in Finland whereas Booth et al. (1996b, 1999b) suggested that appreciable amounts of vitamin K is obtained in the form of dihydrovitamin K_1 in the USA. In addition to low dihydrovitamin K_1 content analysed for margarines, fast food items are not so popular in Finland. It was also suggested that the importance of cereals is insignificant both as source of phylloquinone as well as of dihydrovitamin K_1 despite their overall significance in Finnish diets.

Despite the fairly high average consumption of meat and dairy products in Finland their role as source of vitamin K is only moderate due to their low vitamin K contents. According to this study menaquinones account 90% of total vitamin K intake from animal products, and it seems that chicken meat and cheeses may play a moderate role in vitamin K nutrition. However, the number of samples in this study was not sufficient to evaluate the role of animal products unambiguously. On the other hand, the bioavailability of dietary menaquinones is not known (Vermeer et al., 1995).

This study showed that the significance of plant foods in vitamin K nutrition is high when compared to animal products. However, it has been suggested that bioavailability of vitamin K from different sources varies and for example fat may enhance the absorption of phylloquinone (Gijsbers et al., 1996; Garber et al., 1999). Thus the roles of various food groups cannot be estimated exactly. In addition, the losses occurring during the storage, distribution and cooking were not taken into account in this study. According to studies of

Langenberg et al. (1986) and Ferland and Sadowski (1992b) phylloquinone is quite stable during the heating and freezing process. However, vitamin K is destroyed very easily in light, thus some losses in households during storage and use may occur.

7 CONCLUSIONS

Two HPLC methods for determining vitamin K in foods, one for plant products and another for animal products were developed and validated in this study. Efficient extraction as well as careful purification, especially in the case of animal products with high fat content, was required to quantitatively measure vitamin K contents in foods. HPLC with electrochemical detection was used successfully in analysing phylloquinone contents in plant products. In the case of animal products, the developed method for determining their vitamin K contents was validated and documented in more detail than earlier published methods. The unambiguous identification of menaquinones needed the use of LC-MS in addition to the fluorescence detector. The reliability of the methods was ensured with recovery and repeatability tests, and by participating in collaborative studies.

The dominant vitamin K form in this study was phylloquinone. The role of dihydrovitamin K_1 was estimated to be insignificant in Finland; good estimates of the vitamin K contents of household margarines can be obtained if only phylloquinone is analysed. Although the long-chain menaquinones were found only in a few animal products, the distribution of them varied greatly for example between cheese types. Thus in the case of animal products both phylloquinone and various menaquinones have to be determined.

A reliable database for phylloquinone in plant food items available in Finland was produced. High phylloquinone contents were found in green vegetables, rapeseed and soybean oils and soft margarines. The variation within these food items was remarkable and has to be taken into consideration when planning a sampling system for vitamin K studies. The analysed vitamin K contents in animal products were generally low. More research is, however, needed before their significance as dietary source of vitamin K can be unambiguously evaluated.

The estimated average daily intake of vitamin K in Finland was 120 μg, which is satisfactory when compared to the current recommendation (Food and Nutrient Board, 1989).

Vegetables as well as oils and margarines were the most significant sources. The role of cereals, fruits and berries as well as animal products was minor. Due to the differences in food consumption between individuals and the high variation at least in phylloquinone contents of plant products wide variation in the intake of vitamin K is expected. In addition, the bioavailability of phylloquinone and menaquinones from various sources can vary significantly. On the other hand, the exact requirement of dietary vitamin K is unknown.

This study was one step in increasing our present knowledge about vitamin K in foods. For the majority of the foods the results of this study corresponded well with earlier published values. However, due to variation observed here and also in previous studies in phylloquinone contents, further research, in which the reasons for these variations are investigated, are strongly recommended. The results of this study facilitate planning of sampling for those studies. The methods developed here form a good foundation to further vitamin K research although no universal method for all food items could not be find. In addition, the selection of the internal standard, use of which was necessary due to many steps in sample preparation, is a critical point of consideration. When phylloquinone contents can be analysed reliably with HPLC, further improvements or the use of two detection systems are needed in menaquinone analysis. This study showed the importance of careful documentation of the methods and samples when vitamin K data is produced.

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